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MAGNETIC RESPONSE OF AN ORGANISM AND ITS SOLAR RELATIONSHIPS[1]

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It has become increasingly evident in recent years that the living organism is sensitive to fluctuations in still unidentified geophysical factors, in addition to those factors with which physiologists and ecologists have customarily concerned themselves in the past (Brown, 1959a). Such obvious environmental factors as temperature, light, humidity, gravity, pressure, and sound, are clearly ones toward which it is usually highly important that organisms exhibit immediate adaptive responses. Correspondingly, it is well-known that living things, in general, have their behavior and basic physiology dominated in terms of response to them.

On the other hand, organisms in environments held constant with respect to all these obvious factors still exhibit physiological and behavioral fluctuations correlated with fluctuations in other such geophysical parameters as atmospheric pressure, atmospheric temperature, primary cosmic radiation, and general background radiation, including both their regular atmospheric tidal changes, and their large, weather-related, essentially aperiodic, changes. The correlations are of such character as to indicate clearly that other and yet unidentified factors are also effective for organisms. These less obvious environmental factors appear able to induce or trigger relatively large biological alterations, not uncommonly into the range of 30% or more in their deviation from longer-term mean values. In general, it does not appear adaptive that organisms should permit physiological processes to be regulated by these subtle factors instead of by the more obvious environmental ones, and not unexpectedly, therefore, homeostatic mechanisms seem normally to be continuously operative in reaction to the alterations induced by them.

In two particular associations, however, such subtle environmental factors and their fluctuations may possess clear adaptive value, and there are good reasons to postulate that organisms have evolved physiological means for utilizing this value.

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One of these is in temporal orientation, or the timing of the well-known temperature-independent solar-daily, lunar-daily, synodic monthly, and annual periodicities that commonly persist in complete constancy of all factors to which the organisms are usually deemed sensitive. Indeed, it has been shown possible to account for all the many and peculiar described properties of these long-cycle persistent rhythms in terms of periodicities effected by the subtle factors (Webb and Brown, 1959; Brown, 1959). The second association relates to spatial orientation, particularly as it pertains to the periodic migrations of organisms for feeding and breeding, and especially to the navigational aspects of the phenomenon.

Suggesting a role of subtle environmental factors in animal navigation is the demonstration in recent years of a very close interrelationship between the phenomena of temporal and spatial orientation of animals (Hoffman, 1954; Pardi and Grassi, 1955; Renner, 1959) quite as our own spatial orientation may depend on relationships to sun, moon or the constellations as a function of time. In both of these associations, temporal and spatial orientations, control by the obvious factors, when available, may be the dominating consideration. However, a number of observed characteristics of these phenomena appear inadequately accounted for in these terms alone. Such problems might be greatly simplified were it learned that organisms had available additional information.

On the one hand, it is obvious from available evidence that at least some subtle geophysical factors are perceived by organisms. And yet, on the other hand, cursory examinations for the biological effectiveness of changes in various ones of these within essentially their natural range have in the past yielded generally negative results. Therefore, it seems obvious that the question must be more critically re-examined. The work about to be described is the consequence of a brief, intensive re-examination for possible response to the magnetic field. In this study, reported earlier briefly (Brown, Brett and Webb, 1959; Brown, Bennett and Brett, 1959; Brown, Webb, Bennett and Barnwell, 1959), not only is it apparent that an organism responds significantly to the changes in the magnetic field through alterations in its spatial orientation, but that the response is intimately associated with the mechanism of temporal orientation of organisms quite as might be expected were the magnetic orientation to possess usefulness to the organism.

Methods and Materials

The common mud-snail, *Nassarius obsoleta*, was used in this study. Collections were made at Chappaquoit Beach, West Falmouth, Massachusetts, at approximately weekly intervals, and the stock collections were maintained on a table in running sea-water in the ordinary daily illumination changes of the laboratory.

Simple equipment (Fig. 1) was constructed to determine the degree of right and left turning of snails as they emerged from a narrow corridor into a constant, symmetrically illuminated field. The apparatus consisted of an aluminum, funnel-shaped corral fastened in an 8-inch crystallizing dish set upon a polar-coordinate grid with the opening of the funnel over the center of the grid. A circle of 3 cm. radius was drawn. The grid was ruled into 22.5° sectors, and the long axis of the corridor was accurately aligned with the center of the sector labelled zero. Since the sizes of the snails varied from nearly the width of the corridor to half of that value, even snails following one or the other corridor wall and continuing on a straight path

would, after 3 cm., lie in the zero sector. Successive sectors to the right were given numbers + 1 through + 4, and to the left, − 1 through − 4. This apparatus was placed on the bottom of a 17 × 12 × 10 inch box, as shown in Figure 2, in such a manner that a 4½-inch round window illuminated by an enclosed 60-watt incandescent lamp and covered with a white diffusing transmitter lay above and slightly ahead of the corridor exit. A hooded window for observing the snail movements, and for re-corraling the snails between the successive runs of ten, was

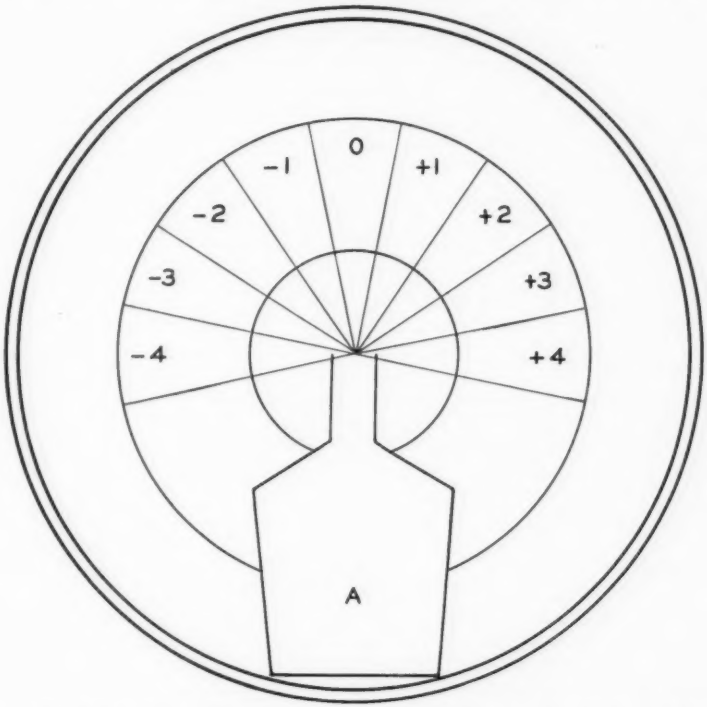

FIGURE 1. Orientation apparatus. View from above of the corral (A) in an 8-inch glass crystallizing dish placed over a polar grid. All dimensions may be scaled from the 3-cm. radius of the innermost circle.

located above and to the rear of the corral. Beneath the box was an adjustable platform with a horizontal turntable upon which an 18-cm. Alnico bar magnet could be easily placed, centered under the corridor exit and orientated in any compass direction, or from which the magnet could be removed quickly. The magnet at 14 cm. below the orientation chamber gave a horizontal intensity (1.5 gausses) only about nine times that of the earth's field in Massachusetts (.17 gauss). Such a weak experimental magnetic field was purposely selected to increase the probability that any response which might be discovered was a naturally occurring phenomenon.

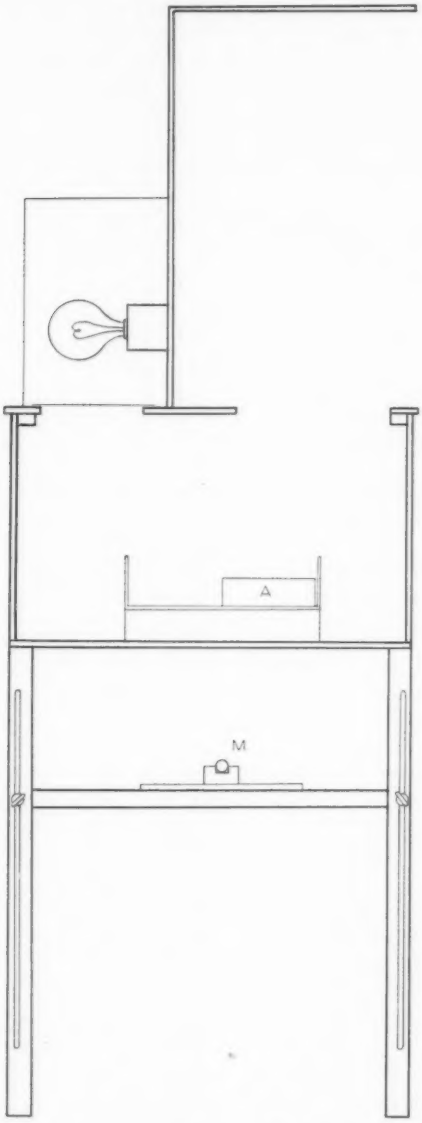

FIGURE 2. Lateral view of the diagrammatically-sectioned wooden orientation apparatus, drawn to scale. A is the corral, M is the bar-magnet.

Such a field strength would be expected to lie within the range of sensitivity of any normally operative responder system which might be present for magnetism. Four identical orientation chambers were available during the study.

The experimental procedures were kept uniform, simple and minimal. The apparatus was always oriented carefully so that the corridor exit was pointed toward the magnetic south. Sea-water to a depth of about 2 cm. was added to the crystallizing dish. Eleven to fifteen snails were dropped into the corral. Each completed experimental series consisted of 60 snail exits, two groups of ten exits without magnet, two groups of ten with the artificially increased magnetic field oriented as the earth's field (with north-seeking pole directed south), and two groups of ten with the north-seeking pole directed west. The first three groups of ten usually consisted of one of each of the three experimental conditions, as did also the last three. The order within the three groups of ten, however, changed continuously. Throughout most of the period of experimentation, the observer was uninformed as to the presence or absence, or orientation, of the magnet. Each snail emergence was accorded one number, that of the sector in which the head, or greater fraction of it, was located at the instant the snail reached the 3 cm. arc.

It is important to note that the method does not involve the determination of any final pathway chosen by the snails; rather, it simply yields a measure of any tendency toward change in direction from the initial path, and the relative degree of this tendency. The use of this kind of measurement appeared to reduce to a minimum obviously erratic, exploratory movements.

It was learned from brief exploratory experiments that the mean path of turning for any given time was not conspicuously influenced by changing the compass direction of orientation of the experimental chamber.

During the two-month period, June 28 to August 29, 1959, inclusive, 564 such series of 60 snail passages were obtained. Series were obtained for all hours of the day from 5 AM through 9 PM, Eastern Standard Time. No single one of these hours of the solar-day was represented by fewer than 9 or by more than 50 series of 60 snail exits.

Results

It was apparent early in the experimentation that the snails, whether controls *without* experimentally imposed magnetic field or experimentals *with* such imposed fields, varied greatly from one time to another in both their spontaneity of activity and their rate of locomotion. This resulted in differences in time for completion of a single series of 60 runs, ranging from about 12 to 60 minutes. Secondly, the pattern of emergences of the snails clearly differed significantly from one time to another under the same experimental conditions. Thirdly, the results of comparisons within single series of 60 runs indicated an influence of the experimental magnetic fields.

Sample patterns of emergence of the snails are illustrated for nine series in Figure 3. These were selected, without reference to time, for the purpose of illustrating the wide variety of results. It is quite evident from this figure that the experimental magnetic fields, even if effective, could not be producing a simple uniform response. As seen from the sample patterns, the introduction of the N–S oriented magnet (B in figure) could, as the average of the two runs of ten snails, be associated with a mean path to left, (*e.g.*, #1, 2, 7, 9) or to the right (*e.g.*, #3,

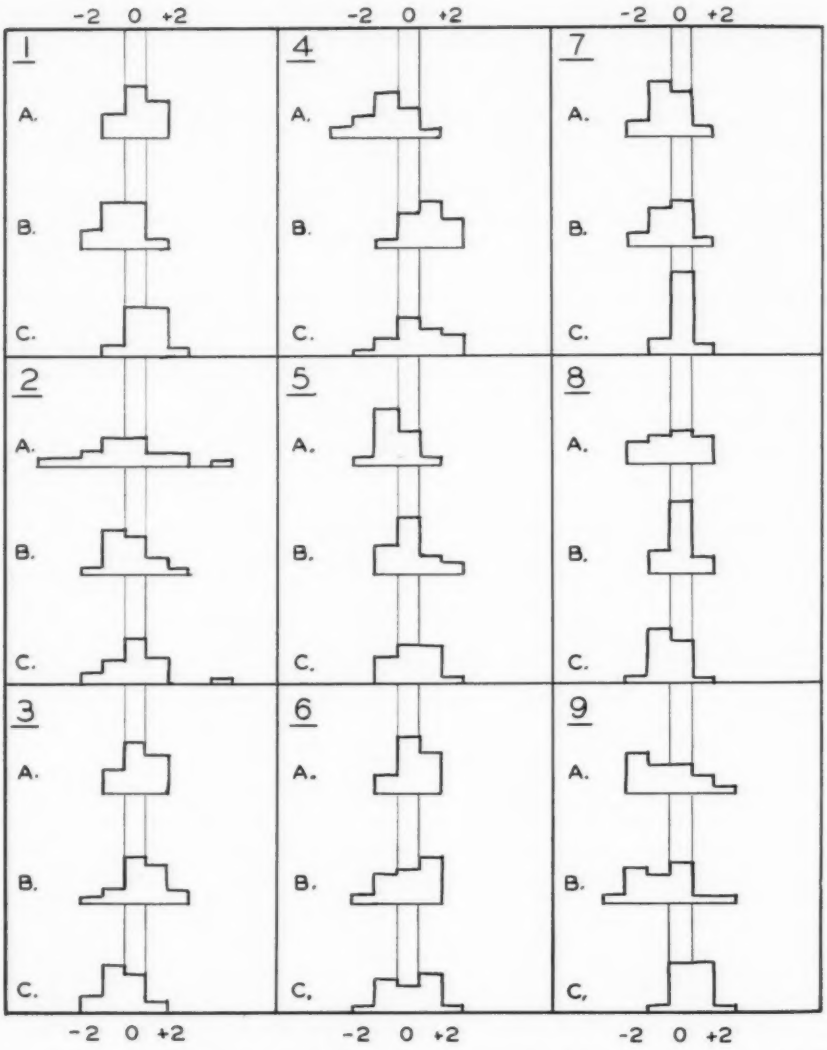

FIGURE 3. The frequency distributions of snail paths found in nine sample series of 60 runs. A, 20 runs in E–W magnet field. B, 20 runs in N–S magnet field. C, 20 control runs only in the earth's (N–S) field. See text for discussion.

4, 8) of the controls. The N–S oriented magnet could appear to effect more dispersion from zero than the controls within the same series (*e.g.*, #3, 7, 9) or less (*e.g.*, #2, 4, 8); it could appear to effect mean paths to right (*e.g.*, #4, 5) or the left (*e.g.*, #1) of the E–W oriented magnetic field (A in figure) or to produce more (*e.g.*, #3, 6) or less (*e.g.*, #2, 8) dispersion than that observed within the same series for the E–W oriented magnet. Comparably, the E–W oriented magnet was associated with patterns to left (*e.g.*, #4, 5, 7, 9) or right (*e.g.*, #3, 8) or with more (*e.g.*, #2, 7, 8, 9) or less (*e.g.*, #3, 6) dispersion than the controls. Ordinary tests for the significance of differences between means, or between standard deviations in the frequency distributions among the three groups of 20 even within these single series, yielded, not uncommonly, probabilities ranging from the 5% to well less than 0.1% level. There was no suggestion that these differences could be explained on the basis of any continuous trend in behavior during the experimental period of a series. In fact, this last possibility was greatly reduced by having each sample of twenty composed of two groups of ten separated by intervening runs under other conditions.

It seemed quite apparent that if a *bona fide* response occurred to either the earth's magnetic field or the experimentally imposed ones, the response could not be simple and invariable. Consequently, analyses of the data involved chiefly three types of values: (a) mean direction and degree of turning; (b) total dispersion as a measure of right and left turning about the zero axis; and (c) standard deviation as a measure of dispersion relative to whatever was the mean path of the given series. The forms of frequency distributions were also always inspected.

(*A*) *Mean path of snails:* Using all data, it became quickly evident that there was a daily rhythm in the average degree of turning of the emerging snails, the animals moving nearly straight ahead at 5 AM and turning increasingly to the left until noon. Thereafter, they turned progressively less, to a second minimum of turning about 7 PM. The mean paths for the snails exposed to the N–S and E–W experimental magnetic fields, and for the controls are plotted as a function of hour of the day in Figure 4A. Indicated also are standard errors of the means for the combined three groups for selected hours. In Figure 4B are illustrated the differences, hour by hour, between the path of the controls and the snails in each of the two experimental magnetic fields. Using all 1128 available differences between experimental and control animals, a mean of -0.0341 ± 0.0104 was obtained. This indicated that the presence of a magnet results in increased left-turning over controls ($P < .005$).

There was no statistically significant difference between the effect of the N–S field (-0.0347) and E–W one (-0.0336). It is, however, suggestive that the 3.2% greater effect of the N–S field is correlated with the actual 12% greater horizontal intensity of the experimental N–S over the experimental E–W field, due to the vectorially additive effect of the earth's natural field in the former experimental condition.

In view of the evident daily rhythm in the mean path observed, together with the suggestion from Figure 4B that increase in strength of magnetic field in the early morning hours yields greater *right* turning, an examination of the effect of the magnetic fields for the hours 7 AM through 9 PM was made. This gave even more decisive indication of increased left-turning in response to the experimental

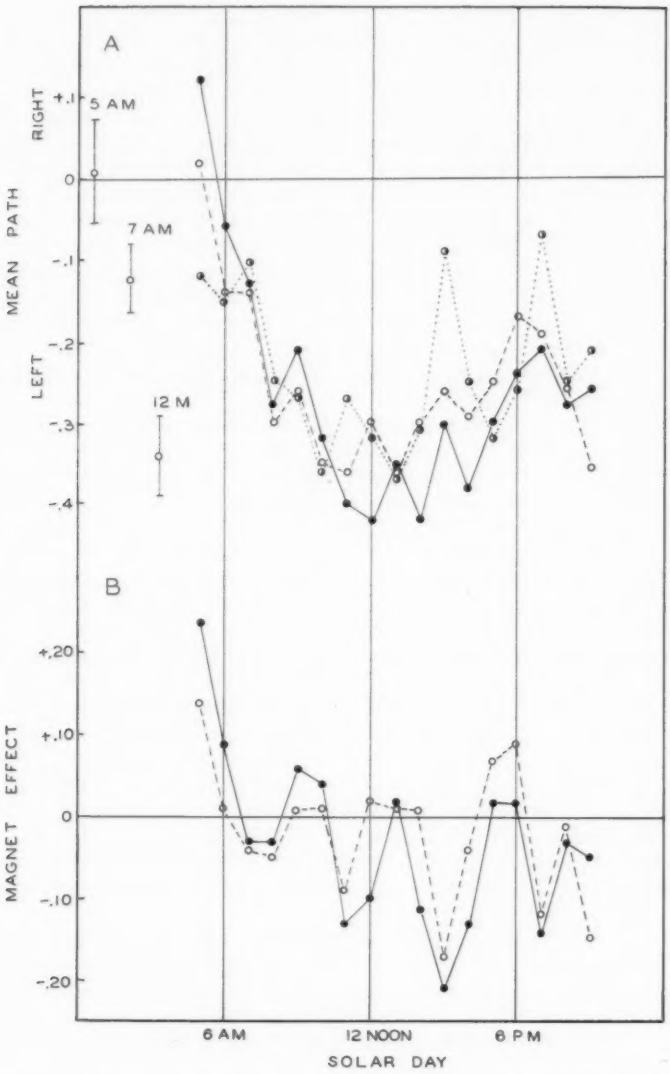

FIGURE 4. A. Mean paths of the snails in the E–W magnet field (dashed lines), in the N–S magnet field (solid line), and control snails (dotted lines) as a function of hour of the solar day. Standard errors of means for all snails are shown for three times of day. B. The difference of mean paths of snails in the E–W magnet field (dashed line) and N–S magnet field (solid line) from the paths of the control snails in the same series.

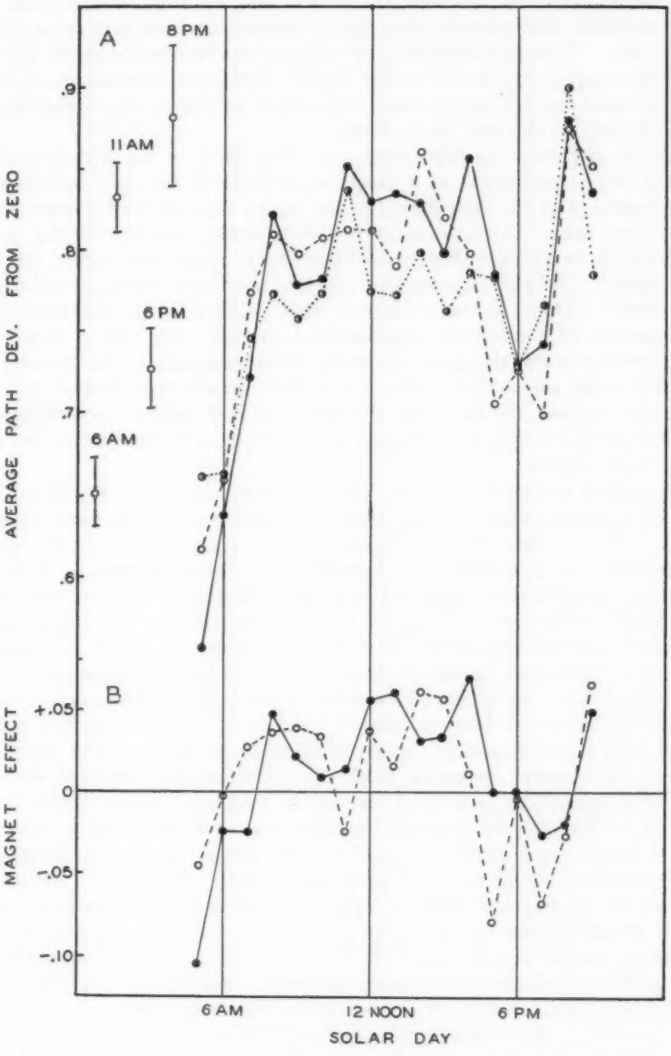

FIGURE 5. A. Mean dispersion of experimental and control snail paths from the zero sector as a function of hour of day. Standard errors of means from all snails are shown for four times of day. B. Difference between experimental snails and controls as a function of time of day. See Figure 4 legend for key.

magnetic fields ($P < .001$). Also, for the 3 PM hour alone the effect of the increased magnetic field strength was highly significantly different from zero ($t = 3.64$; $N = 84$). These probabilities, which leave no reasonable doubt as to a response to the magnet, are found despite highly significant fluctuations of magnetic response of lunar-day frequency which contribute greatly to the variability of the response (Brown, Webb and Brett, 1960).

(*B*) *Mean dispersion of paths from zero:* Inspection of the frequency distributions of the mean snail paths as a function of hours of the day, indicated an increasing bimodality of the distribution of the paths, through a maximum degree of bimodality near noon. A consequence of this is clearly apparent in the form of a daily fluctuation in average dispersion (Fig. 5A). Standard errors for selected hours are shown. A broad maximum is centered over noon, and a second maximum occurs at night. There is also evident a daily rhythm in the difference between mean dispersions of control and experimental animals (Fig. 5B). For a 9-hour period centered at noon, the mean dispersion of the animals as the average for the two magnetic fields was $7.55 \pm 1.96\%$ ($N = 364$) greater than that of the controls. These results suggest, further, that the effect of both magnet positions in early morning and early evening is to reduce dispersion even below that of the controls within the same series.

(*C*) *Standard deviation of paths:* The next analysis was of the fluctuations in standard deviation of paths. It was clear that there was a daily fluctuation in this parameter. This is illustrated in Figure 6A. The significance of this cycle can be seen from the two standard errors included. In this parameter, as in the others, the frequency distributions suggested increasing bimodality for the data obtained over the middle of the day. However, at those times of day, 8–12 AM and 8 and 9 PM, when standard deviations are relatively lower than might be expected in terms of total dispersion (compare Figs. 5A and 6A), the population of values more strongly favors one of the two centers in the bimodal frequency distribution. Figure 6B illustrates the hour-by-hour difference between the control snails and the snails in the experimentally augmented magnetic fields. The daily pattern of effect of the imposed magnetic fields upon altering the standard deviation of paths selected appears to possess at least three maxima. With all the data from the 17 hours of the day, the standard deviation appeared increased by the magnets, though not highly significantly ($P < .05$). For the period 6 AM through 4 PM alone, the statistical significance increased substantially ($P < .01$). However, that an influence of the magnetic fields is being reflected in this parameter is suggested even more strongly from the similarity of the mean daily pattern of effect for the two magnetic orientations, N–S and E–W ($r = 0.72$) ($N = 17$), though the significance of this correlation is obviously tempered somewhat by the fact that there was a common control group for the two experimental groups.

(*D*) *Frequency distribution of magnet responses, relative to hours of day:* In view of the clear indication from the preceding analyses that the imposed magnetic fields effect to various extents either of two types of response, right or left turning, it seems reasonable to presume that (1) the small, but statistically highly significant, predominance of left turning especially between the hours 7 AM and 9 PM, and (2) the clear suggestion of right turning at 5 and 6 AM are simply residuals, a consequence of failure of one response to be cancelled exactly by the

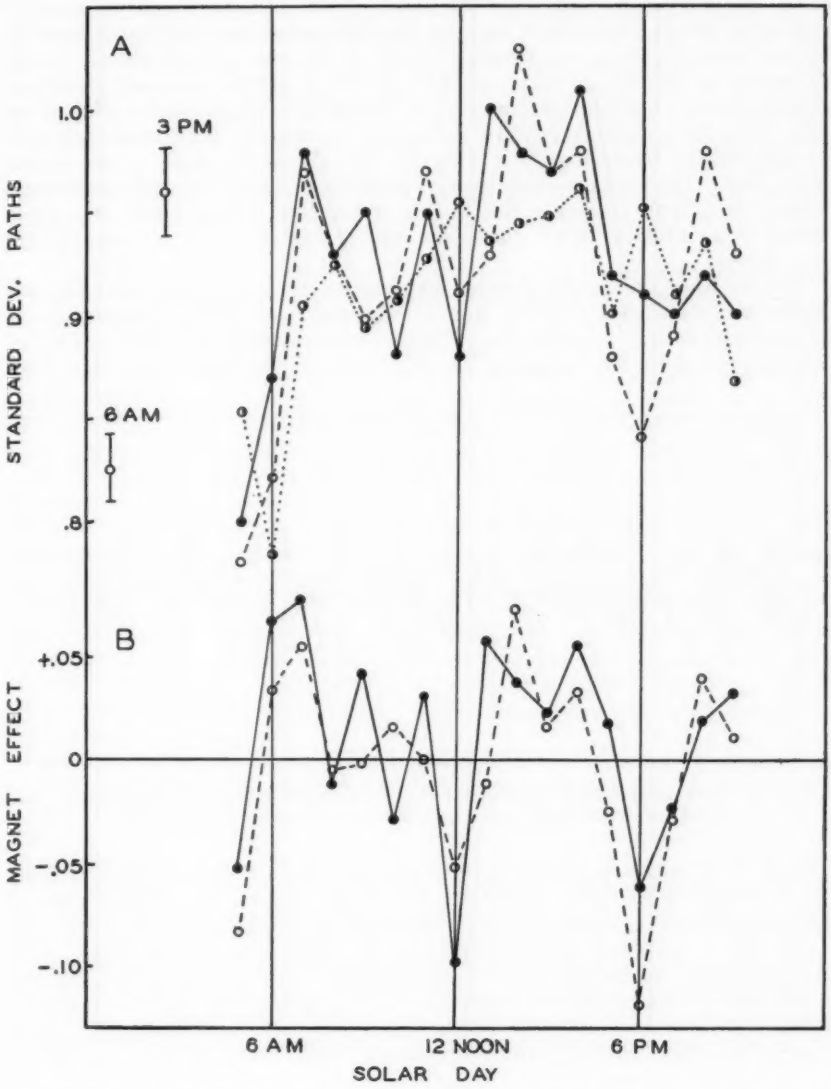

FIGURE 6. A. Standard deviation of experimental and control snail paths, as a function of hour of day. B. Difference between experimental snails and controls as a function of time of day. See Figure 4 legend for key.

other. To cast further light upon this aspect of the problem, frequency distributions of the differences obtained between experimental animals and the controls in the same series are shown as a function of hour of the solar day in Figure 7. From the general dissimilarity of nearly all these to normal distributions, and the strong tendency towards bimodal distributions (see especially 9 AM through 4 PM), these suggest that the organismic response to the imposed magnetic fields is not only real, but substantially larger than was apparent from the simple study of the pooled hourly data. The actual response appears normally to amount to magnetic-induced turning through an average of 0.3 to 0.4, or more, of a sector unit, or a mean turning of the order of 8–10° during the snail passage in a few seconds over the 3-cm. course.

Further inspection of the distributions suggests that a response to the imposed magnet occurs also as a deviation from a mean degree of turning characteristic of the particular hour of the solar day. This is most evident in the 3 PM and 9 PM distributions. It is also suggested for the 5 and 6 AM hours, when the mean

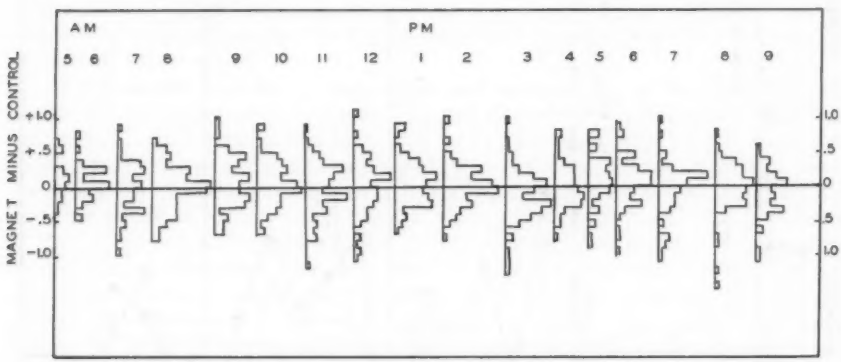

FIGURE 7. Forms of frequency distributions of the differences between all experimental snail paths and control snail paths, as a function of hour of the solar day.

turning of controls is near zero and the response to the magnet is predominantly that of turning to the right. It appears also evident that the mean effectiveness of the two experimental magnetic fields passes through minima at 8 AM, 2 PM, and 7 to 8 PM, as indicated by the reduction in the bimodality at these times. This changing pattern of frequency distributions through the day suggests the presence of two major daily patterns of oscillation in the extent of the response to the experimentally increased magnetic fields, with one pattern essentially the mirror-image of the other, and with cross-over points at these times of minimal response.

DISCUSSION

It is apparent from this study that in the constant symmetrical experimental field the amount of turning of snails is a function of the time of day, and that a daily fluctuation is found in both the mean amount of turning, ignoring sign, and

in the mean path of the snails taking sign into account. This is apparent for all snails, both those in the earth's natural magnetic field and in the imposed fields where the field strength is increased about 10-fold with the direction of magnetic lines of force being left either in the same direction as the earth's, or at right angles to this. The three groups considered as three samples showed remarkable mean similarities as functions of hours of the day, though commonly differing very widely from one another in any individual series (see Fig. 3). This similarity indicated that the amount of right or left turning, or dispersion of paths from zero, was not determined primarily by the direction of the field or by its fluctuations in strength.

Hence, it must be concluded that the response is predominantly a klinokinesis, or a turning relative to any initial compass direction, either clockwise or counterclockwise, as a function of time of day. Of the various possibilities of the factors concerned in the response, three are immediately apparent. (1) The response involves a rate of turning relative to the diffusing light source above and slightly ahead of the path of the snail. This might be interpreted as a move on the part of the snail to assume a particular angular relationship between the long-axis of its body (light-compass relationship) and the artificial gigantic "sun" as a function of the time of solar day; (2) the turning of snails involves a torsional orientation in response to the magnetic field itself as a function of time of day; (3) the orientational response may not be due to any external factor directly, but simply to differential bilateral activity of bodily orientation mechanisms. Or, possibly any two or all might be involved and contribute jointly to the results. However, it is evident from this study that just as would be expected were the response normally one of klinokinesis in response to the magnetic-field, so the klinokinesis can be highly significantly modified through experimentally increased magnetic flux as a function of time of solar day. There also remains an additional possibility, namely, that any light-compass orientation of the snails is, in turn, regulated through an orientation to magnetic field.

Support for the concept that the normal turning of the snail is importantly an orientation in response to magnetic field itself may be seen first in Figure 5. When the dispersion is least in the earth's magnetic field, the effect of increasing magnetic flux is to reduce it still more, and when dispersion is large in the earth's field, the magnet increases it still more. Comparably, as Figure 4 indicates, when left-turning is high in the earth's natural field the magnet increases it, and when it is low, appears to effect turning in the opposite direction, to the right. In a general manner, but slightly more complexly, the relationships of Figure 6 also give a similar kind of support for the view of an important role of the normal earth's field in snail orientation. The effect of the experimentally augmented field is to bring about either further increased, or decreased, size of standard deviation, depending upon which is normally the predominant response characteristic of the time in the daily cycle.

It is very interesting, also, to compare the two aspects of magnetic orientation, left-turning and bimodality of distribution about a mean path, to the two signs of oxidative metabolic fluctuations of *Nassarius* about a mean daily non-inverting fluctuation. Both the non-inverting and inverting components in these metabolic fluctuations appear clearly to be responses to unidentified barometric pressure

correlates (Brown, Webb and Brett, 1959). This suggests an intimate relationship between the mechanisms of temporal and spatial orientation in the snails and points to the possibility that the experimentally augmented magnetic field has in part simply increased the strength of whatever orientational physiological mechanism chanced to be dominant at the time, through a generalized influence upon the mechanism of cellular oxidative energy transformations. For this effect, the magnetic field would need to possess no spatial orientational feature, *per se*. But while this effect might account for the induced increases in standard deviation or increased turning, it could scarcely account for those times of improved precision of orientation by reduction of turning below that of the controls. The last appears more rationally accountable in terms of the concept that the sharp and stronger the magnetic field, the more decisive whatever organismic responses are characteristic of that time of day to it.

Summary

1. The orientation of snails in a constant, symmetrical field was studied over a two-month period, June 28 through August 29, 1959, at various hours of the day between 5 AM and 9 PM.

2. The orientation of snails in the earth's natural magnetic field was compared throughout the study with the orientation of snails subjected to a 9- to 10-fold increase in field strength, with fields both parallel and at right angles to the earth's natural field.

3. A daily rhythm in the direction and average amount of turning was found in the snails; the mean paths of those in the two (N–S; E–W) experimentally augmented magnetic fields were statistically significantly to the left of the controls, particularly between the hours 7 AM through 9 PM.

4. The mean amount of turning, whether clockwise or counterclockwise (klinokinesis), in the experimental magnetic field was also increased significantly over that of controls in solely the earth's field, and similarly exhibited a daily rhythm.

5. Certain similarities between the orientational responses to the magnetic fields and earlier described exogenous metabolic fluctuations in constant conditions, suggest a relationship between them.

6. Evidence is advanced supporting the hypothesis that the orientation of snails normally includes a true response to the earth's magnetic field.

LITERATURE CITED

Brown, F. A., Jr., 1959a. The rhythmic nature of animals and plants. *Amer. Sci.*, **47**: 147–168. (Reprinted from Northwestern Tri-Quarterly, Nov., 1958.)

Brown, F. A., Jr., 1959b. Living clocks. *Science*, **130**: 1535–1544.

Brown, F. A., Jr., M. F. Bennett and W. J. Brett, 1959. Effects of imposed magnetic fields in modifying snail orientation. *Biol. Bull.*, **117**: 406.

Brown, F. A. Jr., W. J. Brett and H. M. Webb, 1959. Fluctuations in the orientation of the mud snail, *Ilyanassa obsoleta*, in constant conditions. *Biol. Bull.*, **117**: 406–407.

Brown, F. A. Jr., H. M. Webb and W. J. Brett, 1959. Exogenous timing of solar and lunar periodisms in the mud snail, *Ilyanassa* (*Nassarius*) *obsoleta*, in laboratory constant conditions. *Gunma J. Med. Sciences*, **8**: 233–242.

Brown, F. A. Jr., H. M. Webb and W. J. Brett, 1960. Magnetic orientation in an organism and its lunar relationships. *Biol. Bull.*, **118**: 382–392.

BROWN, F. A., JR., H. M. WEBB, M. F. BENNETT AND F. H. BARNWELL, 1959. A diurnal rhythm in response of the snail, *Ilyanassa,* to imposed magnetic fields. *Biol. Bull.,* **117**: 405–406.

HOFFMAN, K., 1954. Versuche zu der im Richtungsfinden der Vogel enthaltenen Zeitschatzung. *Zeitschr. f. Tierpsychol.,* **11**: 453–475.

PARDI, L., AND M. GRASSI, 1955. Experimental modification of direction-finding in *Talitrus saltator* (Montagu) and *Talorchestia deshayelsi* (aud.) (Crustacea-Amphipoda). *Experientia,* **11**: 202–210.

RENNER, M., 1959. Über ein weiteres Versetzungsexperiment zur Analyse des Zeitsinnes und der Sonnenorientierung der Honigbiene. *Zeitschr. vergl. Physiol.,* **42**: 449–483.

WEBB, H. M., AND F. A. BROWN, JR., 1959. Timing long-cycle physiological rhythms. *Physiol. Rev.,* **39**: 127–161.

MAGNETIC RESPONSE OF AN ORGANISM AND ITS LUNAR RELATIONSHIPS[1]

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It has been demonstrated (Brown, Brett, Bennett and Barnwell, 1960) that mud-snails, *Nassarius obsoleta,* initially directed magnetic southward in a symmetrical field constant for all factors normally considered able to influence their orientation, exhibit a daily rhythm in the direction of their mean paths. The amount of dispersion of paths of a population of snails about their mean path also displays a daily rhythm. Both of these characteristics of spatial orientation were shown to be quantitatively alterable, in a manner highly significant statistically, by experimentally changing the strength of the ambient magnetic field. Although much of the observed variation, both in the orientation of control animals in the earth's field and in the modified orientation in response to experimentally increased strengths of the magnetic field, was accounted for in terms of the daily rhythm of response, much variation still remained unaccounted for. The following study was made to determine whether lunar periodisms in responsiveness to the magnetic field were also present.

Methods and Materials

The apparatus, and methods of obtaining the data, utilized in this study have already been reported in detail elsewhere (Brown, Brett, Bennett and Barnwell, 1960). In essence, measurements were made of the average amount of clockwise or counter-clockwise turning of snails as they traversed a 3-cm. course immediately following their emergence from a straight, narrow corridor directed toward the magnetic south into an illuminated, symmetrical, constant field. Each of 564 experimental series, obtained during the period June 28 through August 29, 1959, included two groups of exits of ten snails each in the earth's natural magnetic field, two groups of ten in a magnetic field increased 10-fold and oriented as the natural one (N–S), and two groups of ten in a nearly equally strengthened field rotated clockwise through 90° so that the north-seeking pole of the bar magnet was directed west (E–W).

Data were obtained for hours of the day from 5 AM through 9 PM, E.S.T., with no hour of this period represented by fewer than nine series, nor more than 50 series, of 60 snail exits.

Results

Lunar-day rhythm of mean path

All the data on orientation of the snails for each calendar day were rearranged to assume their proper relationship as approximate hours of lunar days. When

[1] This research was aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, #1228-03.

more than one series was recorded for any single hour of a given day, these were combined to yield a single average value. The average direction and amount of turning of the snails as a function of hour of the lunar day is indicated in Figure 1 for the control snails, those in the artificially augmented N–S magnetic field and those in the E–W field. Each of the three differently treated groups is dealt with here as a separate sample. Not only is there a clear mean lunar-day cycle of

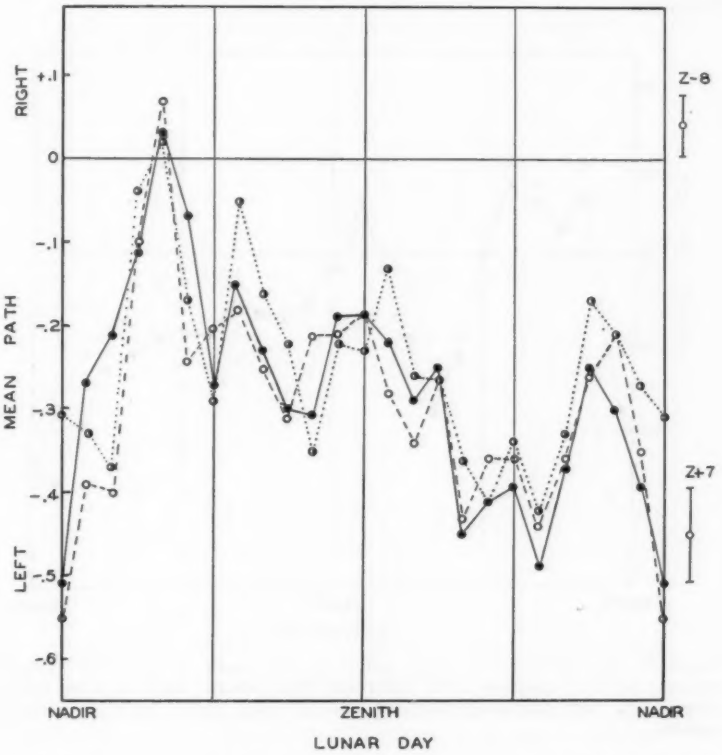

FIGURE 1. The mean paths of snails in the E–W magnetic field (broken line), N–S magnetic field (solid line), and control snails solely in the earth's field (dotted line) as a function of hours of the lunar day. Standard errors of means at two selected times are depicted.

turning, but the three samples exhibit a considerable similarity in the form of the mean cycle despite relatively wide differences between values for the three samples within individual series of 60 (See Brown, Brett, Bennett and Barnwell, 1960).

Not only is the mean lunar-day cycle of turning of essentially the same amplitude as the comparable, previously described solar-day one, but displays a general similarity to it in gross form, though appearing to mirror-image the daily one in

secondary superimposed fluctuations over the period the moon is above the horizon. As with the solar-day cycle, minimum left-turning or maximum right-turning occurs about the fourth hour of the lunar day or near the time of moon-rise, and maximum left-turning occurs at lunar nadir. Standard errors of the means for the fourth (zenith minus 8) and the nineteenth hours (zenith plus 7), calculated from all data for these hours, are indicated on the figure. These values indicate the high degree of statistical significance of this lunar-day fluctuation of mean path of orientation.

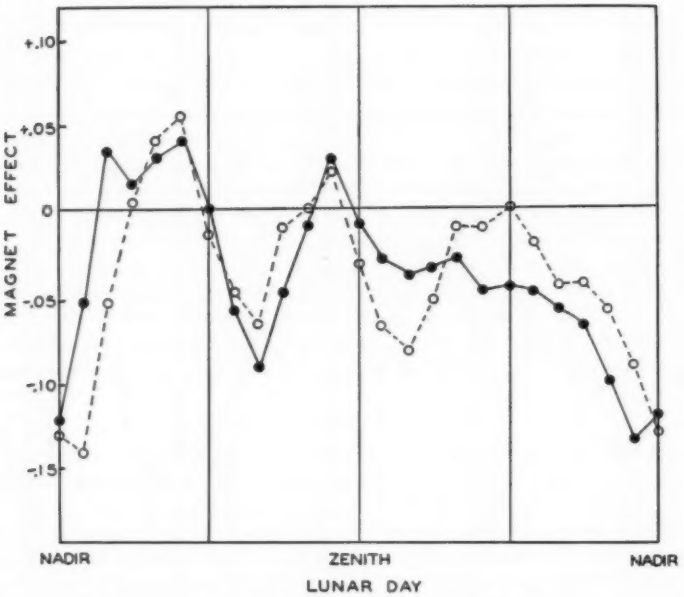

FIGURE 2. The difference of mean paths between snails in the E–W magnetic field (broken line) and N–S magnetic field (solid line) from control snails as a function of hours of the lunar day. The values are calculated from all series studied during overlapping three-hour periods.

Lunar rhythm in effect of experimentally augmented magnetic fields

The average effect of the imposed magnetic fields for overlapping groups of three lunar-day hours were expressed as differences from the comparable three-hour control samples and the mean lunar-day fluctuation in this magnetic effect is plotted in Figure 2. Such three-hour grouping of the data was employed (1) to reduce as far as possible the apparent significance of individual points based upon relatively small numbers of series, and (2) to reduce irregularities in the lunar-day cycle reflecting incomplete randomization of the solar-day cycle as a consequence of the occurrence of occasional solar days lacking in data. As indicated in Figure 2, a maximum right-turning response to the experimental magnetic fields occurs near

the time of moon-rise, and a maximum left-turning response is seen at lunar nadir. This is true whether one deals with either the N–S or E–W experimental fields. The lunar-day pattern suggests three progressively decreasing maxima as one moves through the lunar day. The overwhelming preponderance of negative values, indicating the induction of left-turning by the experimental procedure, clearly corroborates the results of the earlier solar-day study in demonstrating this response to the experimentally imposed weak fields.

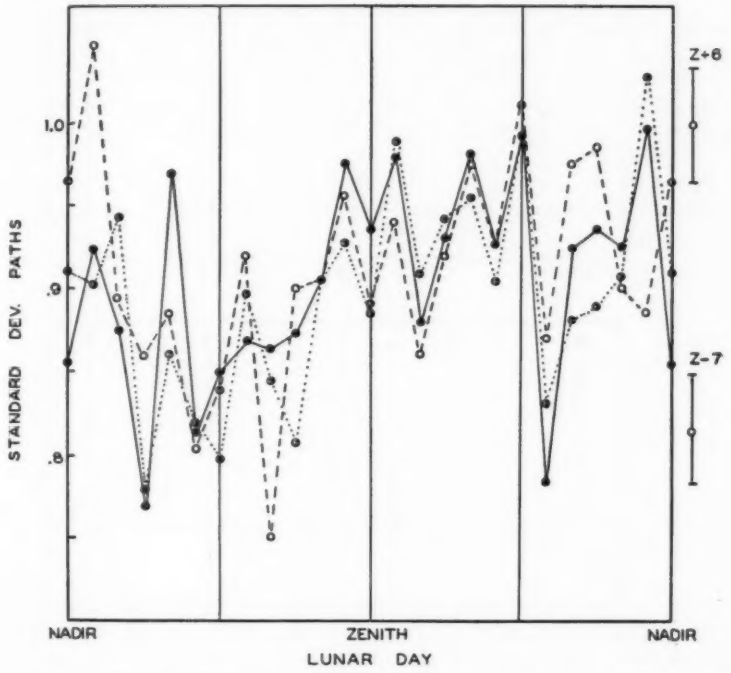

FIGURE 3. The standard deviation of the paths of experimental and control snails as a function of hour of lunar day. Sample standard errors are illustrated. See legend of Figure 1 for key.

Standard deviation of paths

In view of the solar-day rhythm of dispersion of paths established previously and indicating both right and left turning responses to the magnetic field, the standard deviations of all the samples of 20 controls, 20 animals in the N–S experimental field and 20 in the E–W field were next rearranged to ascertain whether a lunar-day fluctuation occurred also in this parameter. In Figure 3 is seen the mean lunar-day fluctuation of standard deviations for the month-period, July 6–August 4, for each of the two experimental conditions and the controls, separately. Standard errors of the means for the seventh hour before zenith and the sixth hour

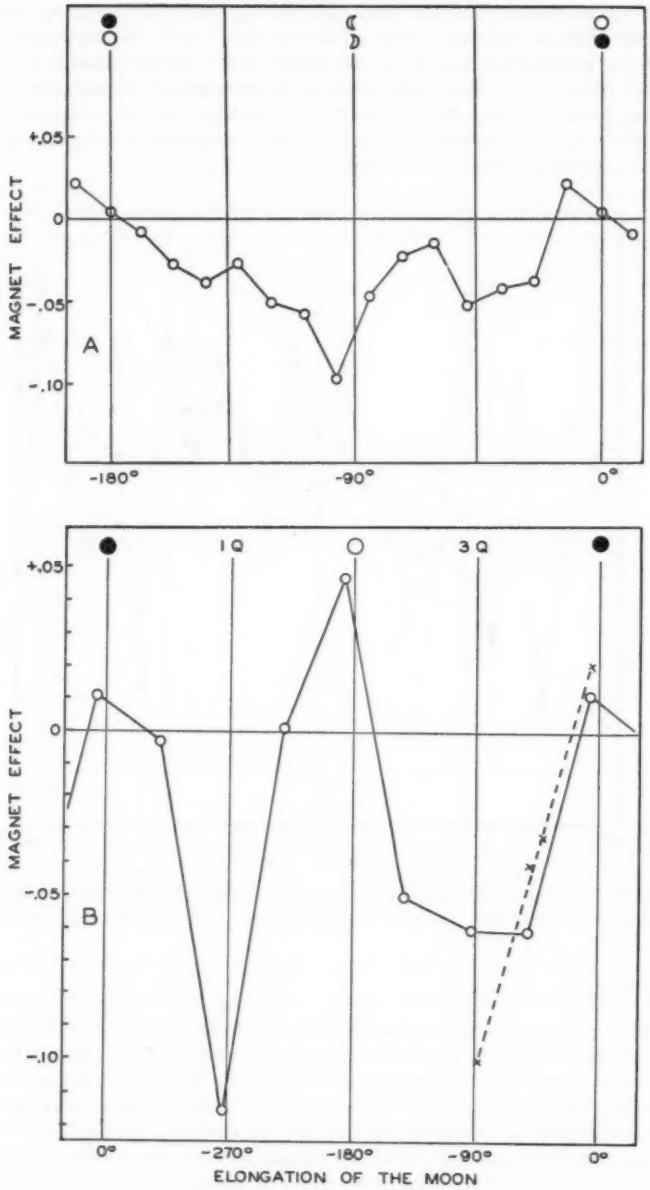
MAGNET EFFECT
+.05
0
-.05
-.10
A
-180°
-90°
0°
1Q
3Q
B
-270°
ELONGATION OF THE MOON

FIGURE 4. A–B.

after zenith indicate the highly significant character of this lunar-day cycle. When, instead, all data for the two-month period were used, this cycle was very similar, but had slightly increased amplitude and statistical significance. However, the particular monthly period was selected since the most complete series of daily studies were conducted during this period, and, hence, more complete randomization of the solar-daily cycle was assured.

Semi-monthly cycle in the mean daily sign and amount of turning

A semi-monthly rhythm of response to the experimental magnetic fields was noted. This was characterized by the experimental snails turning to the right of controls just before each new and full moon, and turning maximally to the left of controls near the times of the moon's quarters. Differences between the paths of experimental snails in the N–S or E–W fields and controls were obtained for a

TABLE I

Mean daily signs of magnetic field responses obtained as a function of day of a natural semi-monthly period

NM or FM	+	+	+	−			
+1	−	−					
+2	+	−	−	−	−	−	
+3	+	−	−	−	−	−	
+4	0	−	−	−			
+5	+	+					
+6	+	−	−	−			
+7	0	−	−	−			
+8	+	+	−	−	−	−	
+9	−	−	−	−			
+10	+	+	−	−			
+11	+	+	−	−	−	−	
+12	+	+	−	−			
+13	+	+	+	+			
+14	+	+	+	+			

total of 64 days during the two-month period of study. The sign of the average response obtained for each of the days on which any series were obtained, as related to the days of new or full moon, is indicated in Table I. In view of the paucity, or absence, of data on some days and the relative abundance on other days it was felt that a more accurate determination of the mean form of this semi-monthly cycle could be obtained by calculating averages of all available series for overlapping periods of three consecutive days each. The results of such a procedure are plotted in Figure 4A.

The values were obtained by considering each day on a semi-monthly frame of

FIGURE 4. A. Semi-monthly cycle in mean daily difference between paths of experimental and control snails. The mean values from overlapping three-day periods of study comprise the points. B. The solid line indicates the fluctuation through the synodic month of the response to the experimental magnetic field based upon 3- to 5-day grouped data, selected to provide mean values at about 45° intervals of elongation of the moon relative to the sun. The broken line shows the relationship after averaging the original data for the two semi-monthly periods, considering both the times of new and full moon as 0°, and using the single range 0° to 90°.

reference of the scales new moon through new moon minus 14 days and full moon through full moon minus 14 days. This provided an arbitrary 15-day semi-monthly period, an adequately close approximation, for the purposes at hand, to the mean, natural 14.8-day one.

In order to assay the statistical significance of this semi-monthly cycle the correlation of the effect of the magnets with the elongation of the moon relative to the sun was assayed. The daily angle of elongation is 12.2°. However, inspection of Figure 4A suggested that the maxima and minima preceded the days of the new and full moon, or its quarters, by about half a day, or by about − 6°. This value, consequently, was treated as the corrected zero one. The data were next combined into eight groups of days in the synodic month: (1) New moon, − 1, and − 2; (2) − 3, − 4, − 5, − 6; (3) − 7, − 8, − 9; (4) − 10, − 11, − 12, − 13, − 14; (5) Full moon −1, − 2; (6) − 3, − 4, − 5, −6; (7) − 7, − 8, − 9; and (8) − 10, − 11, − 12, − 13, − 14. These times were selected to give values centered on times close to those in which the earth-moon and sun-moon axes were parallel with one another (0°), at right angles to one another (90°) and midway between these two (45°). The eight values are plotted in Figure 4B, now corrected for the − 6°. When these were treated as linearly correlated with the angular elongation of the moon corrected to zero at − 6° and one now remained with the 0 to − 90° range of each quadrant of the monthly cycle of lunar elongation, a coefficient of correlation of 0.8486 ($P < .01$) was found. When the same data were further reduced by combining the original data for those pairs of values related to one another by 180°, r became extremely large, 0.9994 (broken line in Figure 4B). This last is obviously a very highly significant value. The substantially lower value of r with N = 8 than with N = 4, in this instance appeared to reflect a quite symmetrical difference between the general forms of the pre-new-moon and pre-full-moon semi-monthly cycles.

The response to experimental magnetic fields appears definitely correlated with the elongation of the moon, and in a rather regular manner. However, the restrictions in the number of values used in the reduced data clearly do not permit one to conclude with any certainty that the correlation is a truly linear one.

Synodic monthly fluctuation in mean daily standard deviation of paths

In Figure 5A are plotted together the mean daily standard deviations of paths of the controls, and the experimental snails in the N–S and E–W magnetic fields. Each is treated separately. During the first forty days of the study there appears to be a synodic monthly fluctuation in standard deviation, with the maximum deviation about two days prior to new moon and minimum deviation about the time of full moon. The last 13 days of data are not in any fundamental manner incompatible with the view of a continuing monthly periodism of the same general character, but do contain, over a short period, some tremendous fluctuations in the standard deviations which clearly can not themselves be part of a simple monthly cycle of the relative regularity of the first. This suggests significantly differing responses of the snails of an aperiodic character, in the constant conditions.

Figure 5B is a plot of the mean daily barometric pressure values for the corresponding period of time. The barometric pressure changes appear related more

than coincidentally to the fluctuations in standard deviations of the snail orientation. Not only are the snail orientation and the barometric pressure fluctuations the mirror-images of one another in gross general trends, but when the barometric pressure fluctuation is treated as a two-day lead correlation on snail orientation,

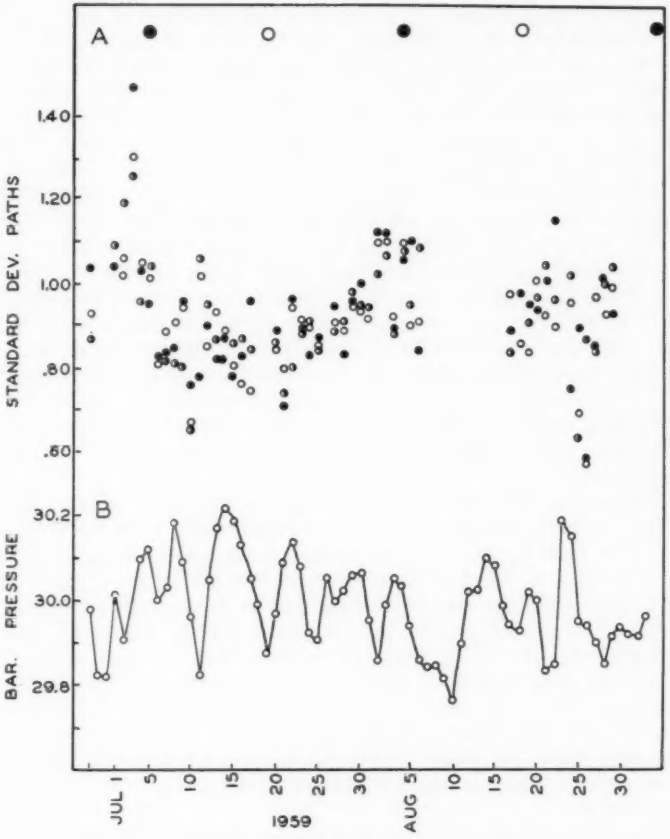

FIGURE 5. A. The mean daily standard deviations of all snail paths for the whole period of study. Open circles, snails in E–W magnetic field; closed circles, in N–S magnetic field; half open circles, controls. B. Mean daily barometric pressure for the period corresponding to (A).

even a number of the major irregularities in the fluctuations of snail orientations, and particularly those of the last thirteen days, become strikingly accounted for in these terms. The calculated two-day lead correlation was $r = 0.33 \pm 0.08$ ($N = 141$), clearly highly significantly different from zero.

Discussion

There appears little reason to doubt that spatial orientation of snails, expressed as an amount of turning, or a klinokinesis, possesses a lunar rhythmicality. This is displayed first as lunar-daily, or approximately 24.8-hour, periodicities in both mean orientational paths with signs taken into account and total turning, both clockwise and counterclockwise. The latter is indicated here in terms of standard deviation of pathways. It is interesting that the form of the lunar-day cycle, in the range of hours corresponding roughly with those available for the solar-day study, namely 5 AM through 9 PM, is strikingly similar to that of the solar-day one in both gross features and phase relationships. At both the times of sun-rise and moon-rise the left turning is minimal and it generally increases towards the early solar and lunar "evenings,"—*i.e.,* times of setting of sun or moon. Also, in both the solar and lunar days standard deviation of pathways is minimal about the time of sun-rise and moon-rise, and increases systematically while the sun and moon are above the horizon, decreasing again as these heavenly bodies set. Furthermore, the amplitudes of the solar and lunar cycles are of the same general magnitude. The presence of these two similarly conspicuous periodisms would be expected to give rise to a synodic monthly cycle, and such a cycle appears to be present. The occurrence of a semi-monthly cycle of influence of the experimental magnetic fields suggests that either the lunar-day or solar-day cycle of mean path is bimodal. Though the former is suggestive of bimodality, the complete form of the solar-daily cycle has not been determined. A semi-monthly rhythm would conform to that of the recurrence of the tides at a particular hour of the day in the natural habitat.

It is evident that since the snails were always subjected, whether in the field or laboratory, to daily illumination changes, a solar-daily cycle could be imparted to the snails by light cycles. The lunar-day period, on the other hand, could be gained by the snails in the field from the tides, but during the periods of as long as 8 to 10 days when the snails were retained in the laboratory any exogenous lunar period in terms of obvious environmental factors was not available. In view of the persistence of the lunar-day rhythm with conspicuousness equal to that of the solar-daily period, it would appear probable that both the lunar-day cycle and solar-day cycles are being derived continuously in response to some unidentified subtle environmental factors in a manner comparable to that demonstrated for the solar and lunar periodisms in *Uca pugnax* (Webb and Brown, 1958; Brown, 1959) and of the metabolism of *Nassarius* itself (Brown, Webb and Brett, 1959).

The similarities between barometric pressure changes and certain parameters of spatial orientation of snails suggest strongly that orientation in the snails is in some manner dependent upon subtle environmental factors other than magnetic fields and that these other factors may also induce considerable alteration in snail behavior. This is indicated, since even the experimentally augmented magnetic fields may be overriden by the factor correlated with the barometric pressure. One possible factor which has been suggested is electrostatic field (Webb, Brown and Brett, 1959). The correlation with barometric pressure recalls the correlation observed for metabolic fluctuations in the snails in constant conditions, including pressure, during the summer of 1958 (Brown, Webb and Brett, 1959), except

for its being a mirror-image. Since there is reason to believe (Brown, Brett, Bennett and Barnwell, 1960) that the spatial orientation described in this study is at least in some measure a klinokinetic response to the natural and experimental magnetic fields, it is possible that fluctuations in other subtle geophysical factors can influence the sensitivity of the magnetic responder-system. However, the possibility can not be excluded that a responder system for another factor is being altered by the magnetic field changes. The apparent effectiveness of certain barometric pressure correlates in modifying the orientational responses, and the well-established role of these correlates in influencing the rate of cellular oxidations, suggest that the sensitivity to the magnetic field is in some manner related to rate of oxidative metabolic changes.

These striking relationships between strength and character of magnetic orientation and phases of the lunar-day and synodic monthly periods contribute, together with the earlier demonstrated solar-day relationships, a substantial degree of predictability to the responses of the snail to magnetic forces. Collectively they increase still more not only the probability that this magnetic response is real, but that it plays a significant role in both the lunar-dominated tidal and the solar-day periodic behavior of these animals.

It is interesting, further, to note that in the lunar-day fluctuation in orientation, with sign taken into account, those times of lunar day when the mean path of the snails is essentially straight or even slightly to the right, the experimentally augmented magnetic field effects turning to the right, and as the snails normally orient during the lunar day more and more to the left as one approaches lunar nadir, the effect of the increased magnetic field is to produce progressively stronger left-turning. This relationship is quite comparable to that found for the solar day and provides further support for the view that response to the earth's natural field normally occurs.

There has been noted a remarkable similarity in both the gross form and the detailed trimodal character of progressively decreasing maxima of the lunar-day, N–S magnetic field responses of snails and the mirror-image of a highly significant mean lunar-day cycle of spontaneous motor activity found in white mice over the 5-month, partially overlapping period, March through July, 1959 (Terracini and Brown, unpublished) similarly treated as a three-hour moving mean. The maxima and minima were essentially synchronous when the two cycles, studied in two places, Massachusetts and Illinois, were adjusted to simultaneity (universal time). The coefficient of correlation for the two trimodal cycles was -0.85. Similarly high simultaneous correlations have been reported earlier between fluctuations of the nucleonic component of cosmic radiation in Illinois and fiddler crab and sea-weed metabolism in constant conditions in Massachusetts (Brown, Webb and Bennett, 1958). This similarity between the cycles of mice activity and response of snails to the magnetic field supports the hypothesis that in this magnetic response we are dealing with a widely, probably universally, occurring biological phenomenon.

The proof of a remarkable sensitivity of an organism to one subtle environmental factor (magnetic field) operating at an intensity only slightly above the earth's natural one, and the evidence for its modification in these constant conditions by fluctuations in a second natural factor, poses even the very fundamental problem

of the dispensability, or indispensability, of the earth's milieu of subtle factors and its natural fluctuations, as a normal ecological consideration in organismic survival and species propagation.

Summary

1. The direction, and mean amount, of turning in snails initially directed southward into a constant symmetrical, illuminated field displays a lunar-day rhythm with minimum turning about the time of moon-rise, and maximum turning at lunar nadir. There is also a lunar-day cycle of standard deviation of snail pathways, with a minimum about moon-rise and a maximum near moon-set.

2. The response of snails to an experimentally augmented magnetic field also exhibits a lunar-day rhythm with maximum turning to the left at lunar nadir.

3. The specific character of the lunar-day rhythm of the response to the experimental magnetic fields gives further support for the view that magnetic field is normally involved in snail orientation.

4. The mean daily response of snails to experimental magnetic fields, expressed as differences from the response of controls in the earth's natural field, displays a semi-monthly rhythm. Maximum right-turning in response to a magnetic increase of 10-fold over that of the earth occurs one to two days before new and full moon, and maximum left turning just before the times of the first and third quarters of the moon.

5. There is a synodic monthly fluctuation in mean daily standard deviations of snail paths with maximum deviation about two days before new moon and minimum deviation about the time of full moon.

6. Some suggestive correlations are demonstrated between barometric pressure and the spatial orientation of snails in an environment constant with respect to all generally accepted orienting factors.

7. It is pointed out that similarities in influence of some unidentified barometric pressure correlates on (a) magnetic orientation of snails, (b) general cellular oxidations, and (c) spontaneous activity cycles present reasons for postulating that the latter two phenomena are in some manner related to the magnetic field response and suggest that response to magnetic field is a widely distributed biological phenomenon.

8. Evidence is presented that suggests there is a biological influence of a universal-time-related, rhythmic, environmental factor.

LITERATURE CITED

Brown, F. A., Jr., 1959. Living clocks. *Science*, **130**: 1535–1544.

Brown, F. A., Jr., W. J. Brett, M. F. Bennett and F. H. Barnwell, 1960. Magnetic response of an organism and its solar relationships. *Biol. Bull.*, **118**: 367–381.

Brown, F. A., Jr., H. M. Webb and M. F. Bennett, 1958. Comparisons of some fluctuations in cosmic radiation and in organismic activity during 1954, 1955 and 1956. *Amer. J. Physiol.*, **195**: 237–243.

Brown, F. A., Jr., H. M. Webb and W. J. Brett, 1959. Exogenous timing of solar and lunar periodisms in metabolism of the mud-snail, *Ilyanassa* (*Nassarius*) *obsoleta*, in laboratory constant conditions. *Gunma J. Med. Sci.*, **8**: 233–242.

Webb, H. M., and F. A. Brown, Jr., 1958. The repetition of pattern in the respiration of *Uca pugnax*. *Biol. Bull.*, **115**: 303–318.

Webb, H. M., F. A. Brown, Jr. and W. J. Brett, 1959. Effects of imposed electrostatic fields on rate of locomotion in *Ilyanassa*. *Biol. Bull.*, **117**: 430–431.

INVESTIGATION OF THE HORMONES CONTROLLING THE DISTAL RETINAL PIGMENT OF THE PRAWN PALAEMONETES [1]

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The distal retinal pigment of the prawns *Palaemon* and *Palaemonetes* has been observed after appropriate stimulation in the fully light-adapted position and the fully dark-adapted one as well as in an intermediate state (Kleinholz and Knowles, 1938; Sandeen and Brown, 1952). The position of the distal pigment was a function of the brightness of the visual field.

Migration of the distal pigment is mediated by blood-borne substances. Kleinholz (1936) found that extracts of eyestalks from *Palaemonetes* caused this pigment to migrate toward the fully light-adapted position. Brown, Hines and Fingerman (1952) obtained the same response with extracts of supraesophageal ganglia. The latter investigators also provided indirect evidence for a hormone that would move the distal retinal pigment toward the fully dark-adapted position. Direct evidence for a dark-adapting hormone in *Palaemonetes* was supplied by Fingerman, Lowe and Sundararaj (1959). When extracts of eyestalks were injected into prawns whose distal pigment was in a position approximately midway between the fully light-adapted and dark-adapted states, a light-adaptational response occurred and lasted about two hours. A dark-adaptational response that lasted approximately five hours followed the light-adaptational one. The supraesophageal ganglia appeared to contain light-adapting hormone alone. No dark-adaptional response was obtained with extracts of eyestalks that was not preceded by a light-adaptational one.

The major objectives of this investigation carried out on *Palaemonetes* were two-fold. The first aim was to learn if the amount of light-adapting hormone in the supraesophageal ganglia could be altered by maintaining specimens in light and in darkness. This aspect was part of a continuing investigation in our laboratory of the effects of long-term adaptation upon endocrine sources in crustaceans. The second objective was to effect at least a partial separation of the distal retinal pigment light-adapting and dark-adapting hormones from each other.

Materials and Methods

Two species of prawns, *Palaemonetes vulgaris* and *P. pugio*, collected in the region of Woods Hole, Massachusetts, were used in this investigation.

The method used to determine the position of the distal retinal pigment was a slight modification of that devised by Sandeen and Brown (1952). The prawns were placed one at a time, ventral surface down, on the stage of the stereoscopic

[1] This investigation was supported in part by Grant No. B–838 from the National Institutes of Health.

dissecting microscope. With the aid of an ocular micrometer and transmitted light the following measurements were made: (1) the width of the translucent portion of the compound eye in a plane parallel to the long axis of the eyestalk, and (2) the length of the eye from the corneal surface to the distal edge of the dorsal pigment spot at the base of the eye proper. Sandeen and Brown (1952) had measured to the proximal edge of this pigment spot. To render the distal clear portion of the eye more translucent and the proximal edge of this area more definite, the prawns were submerged in a dish of sea water on the stage of the microscope. The ratio of width of clear area (measurement 1) to total length (measurement 2) will be referred to as the distal retinal pigment index. Use of this ratio minimized the effect on the data of size differences. In a fully dark-adapted eye the distal retinal pigment abutted against the cornea, the mean distal retinal pigment index was 0.00. In a fully light-adapted eye the distal pigment index was about 0.25.

For all experiments wherein prawns were injected with tissue extracts the assay animals were placed into black enameled pans containing sea water about one inch deep. The pans were then placed at least one hour before the animals were injected under an illumination of such intensity that the distal retinal pigment was approximately midway between the fully light-adapted and dark-adapted positions. The light intensity used in each experiment will be stated below. Each extract was assayed on 10 prawns and the dose was 0.02 ml. per specimen. The assay animals had had one eyestalk removed at least 12 hours before use in an experiment. Because the eyestalk contained a large quantity of light-adapting hormone, one-eyed prawns would not be as readily able to antagonize injected dark-adapting hormone as would intact specimens.

Student's t test was used for determination of the level of significance between means. The 5% level was considered the maximum for a significant difference. Standard deviations and standard errors for the differences between means were also calculated. The results of the statistical analyses are summarized in Table I.

A Model E-800-2 Filter Paper Electrophoresis Apparatus manufactured by the Research Equipment Corporation, Oakland, California, was housed in a constant temperature room maintained at 43° F. on the days the electrophoretic analyses reported below were performed. For each experiment 40 eyestalks were extracted in 0.2 ml. distilled water. The extract was then centrifuged and applied with the aid of a hot air blower across a one-half-inch-wide Whatman No. 1 filter paper strip. The region of application was never more than ¼ inch long. This strip was then moistened with 0.1 *M* sodium hydroxide-boric acid buffer of pH 9.0 and placed into the migration chamber. After electrophoresis had proceeded for two hours at 500 V. and 0.1 mA. the strip was removed from the chamber. The region of application of the extract and one three-inch section from each side of the origin were placed into individual covered dishes containing 0.3 ml. sea water. These dishes were kept in the cold room for 30 minutes to allow materials to wash from the filter paper. As a control in the electrophoretic analyses whenever a strip was removed from the migration chamber a strip of filter paper three inches long was moistened with buffer and placed into 0.3 ml. sea water for 30 minutes in the cold room. The fluid from the containers was then collected in syringes for injection into assay animals.

TABLE I

Summary of the statistical analyses of the data presented herein. N, number of distal pigment indices used in the analysis; S.D., standard deviation; S.E., standard error of the difference between the means; t, Student's t; p, probability value. See text for complete explanation.

Analysis	N	Mean	Range	S.D.	S.E.	*t*	*p*
1 V	43	0.110	0.05–0.22	0.0356	0.00739	0.91	0.40
P	39	0.116	0.05–0.18	0.0326			
2 V	20	+0.034	(−0.02)–(+0.11)	0.0319	0.01044	0.73	0.50
P	20	+0.041	(−0.04)–(+0.11)	0.0340			
3 E	30	0.221	0.14–0.30	0.0468	0.0114	9.66	0.001
C	30	0.136	0.09–0.23	0.0395			
4 E	29	0.159	0.10–0.28	0.0363	0.00835	2.33	0.05
C	30	0.136	0.09–0.23	0.0268			
5 L	95	0.142	0.05–0.28	0.0376	0.00714	5.52	0.001
D	88	0.188	0.09–0.30	0.0519			
6 S	28	0.162	0.03–0.28	0.0754	0.0176	3.53	0.01
F	9	0.253	0.18–0.29	0.0309			
7 E	48	0.249	0.11–0.33	0.0578	0.01022	6.10	0.001
C	54	0.180	0.10–0.25	0.0432			
8 E	112	0.087	0.03–0.23	0.0311	0.00436	14.7	0.001
C	95	0.135	0.07–0.21	0.0299			
9 E	55	0.224	0.11–0.38	0.0474	0.00827	7.24	0.001
C	48	0.131	0.03–0.23	0.0362			
10 E	75	0.079	0.03–0.21	0.0311	0.00514	6.02	0.001
C	87	0.109	0.05–0.21	0.0342			
11 W	55	0.224	0.11–0.38	0.0474	0.00953	2.81	0.01
T	55	0.185	0.10–0.29	0.0524			
12 E	62	0.086	0.03–0.17	0.0359	0.00622	2.49	0.02
C	68	0.104	0.05–0.17	0.0348			

Analysis 1, Background adaptation of *P. vulgaris* (V) and *P. pugio* (P).
Analysis 2, Light-adapting potency of supraesophageal ganglia of *P. vulgaris* (V) and *P. pugio* (P).
Analysis 3, Supraesophageal ganglia of specimens two weeks in darkness (E) versus control (C).
Analysis 4, Supraesophageal ganglia of specimens two weeks in light (E) versus control (C).
Analysis 5, Supraesophageal ganglia of prawns in light (L) versus supraesophageal ganglia of prawns in darkness (D).
Analysis 6, Distal pigment index at start (S) and finish (F) of experiment.
Analysis 7, Eyestalk light-adapting hormone (E) versus control (C).
Analysis 8, Eyestalk dark-adapting hormone (E) versus control (C).
Analysis 9, Eyestalk light-adapting hormone (E) versus control (C).
Analysis 10, Eyestalk dark-adapting hormone (E) versus control (C).
Analysis 11, Comparison of light-adaptation produced by extracts of eyestalks without trypsin (W) and with trypsin (T).
Analysis 12, Dark-adaptation after electrophoresis of eyestalks (E) versus control (C).

Experiments and Results

The distal retinal pigment of P. pugio and P. vulgaris

The specimens of *Palaemonetes* in the Woods Hole area had always been considered to be *P. vulgaris* (*cf.* Allee, 1923). However, Holthuis (1952) separated the prawns of the Woods Hole area into three species, *P. vulgaris, P. pugio* and *P. intermedius*. No investigator has reported specific differences of retinal pigment cells in *Palaemonetes*.

Examination of 451 specimens used in the present investigation revealed the presence of specimens of two of the species only, *P. vulgaris* and *P. pugio*. An overwhelming percentage (92.0%) was *P. vulgaris*. The present investigators used *Palaemonetes* without regard to species. This procedure was justifiable because the following two experiments revealed that the distal retinal pigments of *P. vulgaris* and *P. pugio* were physiologically indistinguishable.

(1) This experiment was designed to answer the following question. Are the mean distal retinal pigment indices of specimens of *P. vulgaris* and *P. pugio* exposed to the same intensity of illumination substantially the same? Forty-three specimens of *P. vulgaris* and 39 of *P. pugio* were placed into black enameled containers under an illumination of 31 ft. c. After one hour the distal retinal pigment index of each specimen was determined. The mean distal pigment indices were 0.110 for *P. vulgaris* and 0.116 for *P. pugio*. Statistical analysis of these data revealed no significant difference between the means (Table I, Analysis 1).

(2) The next experiment answered two questions. Do differences exist between the light-adapting potencies of extracts of supraesophageal ganglia from the two species? Are the responses of specimens of both species to the same extract different? Extracts with a concentration of 10 organs per ml. of sea water were prepared from supraesophageal ganglia of *P. vulgaris* and *P. pugio* and were assayed on 10 specimens of each species. The assay animals were in black enameled pans under an illumination of 31 ft. c. As a measure of the light-adapting potency of each extract, activity (potency) values were calculated in the following manner. The sum of the mean distal pigment indices, determined 30 and 60 minutes after the prawns were injected with the extracts, was subtracted from the sum of the means of the indices determined at the same time intervals with control specimens of the same species as the prawns injected with the extract of supraesophageal ganglia. The controls were injected with sea water. This difference between the sums was a measure of the potency of the extract. Thirty and 60 minutes were chosen because maximal light-adaptation in response to extracts of eyestalks and supraesophageal ganglia always occurred 30–60 minutes after injection. The activity values were as follows: "vulgaris" supraesophageal ganglia into "pugio," 0.068; "vulgaris" into "vulgaris," 0.068; "pugio" into "vulgaris," 0.076; "pugio" into "pugio," 0.082. An extract made from the supraesophageal ganglia of animals randomly selected from the stock aquaria and assayed on randomly selected assay animals was 0.071. The "vulgaris" into "pugio" and "pugio" into "pugio" potency values were analyzed statistically to determine if the difference between them was significant. These values were chosen because they were at the extremes. For the statistical analysis the mean distal pigment index of the control specimens was subtracted from each index obtained

from prawns injected with the extracts. The algebraic sign was noted. As anticipated, the two sets of data were not significantly different from each other (Table I, Analysis 2).

Influence of light and darkness upon the amount of light-adapting hormone in the supraesophageal ganglia

One group of prawns was placed in a darkroom, a similar group was placed into white enameled pans and exposed to a constant illumination of 31 ft. c. After 14 days the supraesophageal ganglia of specimens of both groups were ex-

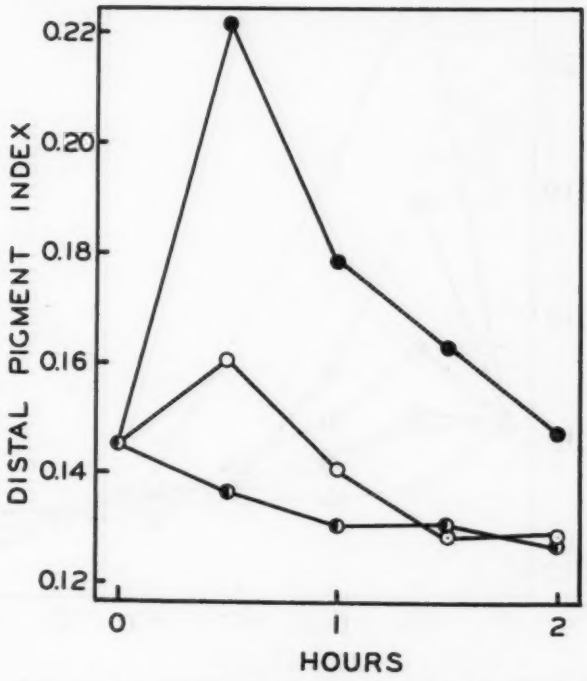

FIGURE 1. Responses of the distal retinal pigment to extracts of supraesophageal ganglia of prawns maintained on a white background under an illumination of 31 ft. c. (circles) and in darkness (dots) for 14 days. Control, half-filled circles.

tracted in sea water (10 organs/ml.) and assayed on one-eyed prawns in black enameled pans under an illumination of 31 ft. c. Control specimens received injections of sea water.

Extracts of the supraesophageal ganglia of both groups produced a light-adaptational response. However, the responses of the assay animals to the organs of specimens that had been in darkness was much greater than that produced by the extracts of supraesophageal ganglia of specimens that had been

illuminated. The experiment was done two more times. The results were qualitatively the same as those obtained the first time the experiment was done. Data of the three experiments were consequently averaged and the means were used in the preparation of Figure 1. The light-adaptational responses of both groups of assay prawns were statistically significantly different from the controls

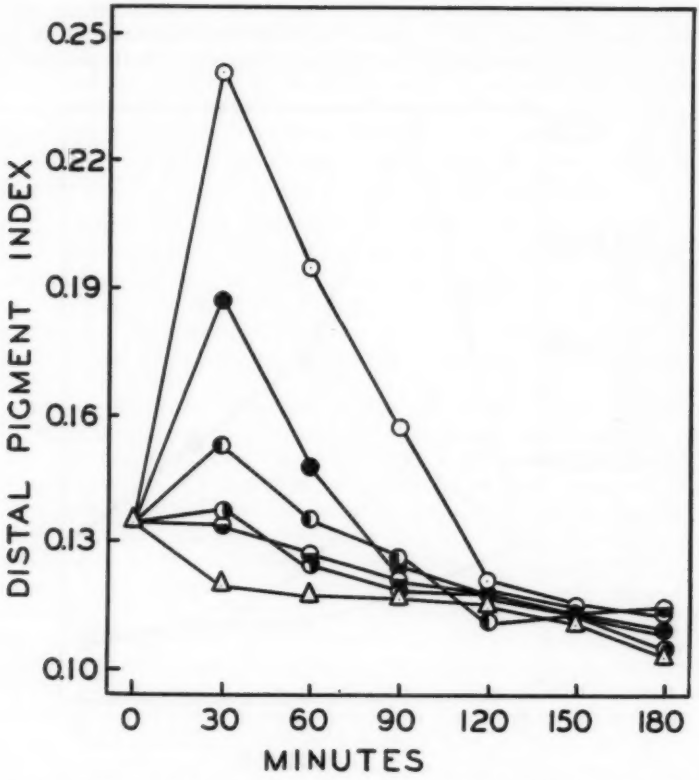

FIGURE 2. Response of the distal retinal pigment to several concentrations of extracts of supraesophageal ganglia of animals collected less than 24 hours before use. Circles, one organ per 0.02 ml. sea water; dots, ½ organ per 0.02 ml.; circles half-filled on left, ¼ organ per 0.02 ml.; circles half-filled on right, ⅛ organ per 0.02 ml.; circles half-filled on bottom, 1/16 organ per 0.02 ml.; triangles, control of sea water.

(Table I, Analyses 3, 4). The distal pigment indices determined 30 minutes after the extracts had been administered were used in the statistical analyses. The difference between the responses of assay animals to the extracts of the supraesophageal ganglia of the specimens that had been in light and in darkness was also highly significant (Table I, Analysis 5). Analysis 5 was based on the

distal pigment indices determined 30, 60 and 90 minutes after the extracts had been injected.

The next experiment was designed to learn how the amount of light-adapting hormone in the supraesophageal ganglia of freshly collected specimens compared with the amounts of this hormone in the supraesophageal ganglia of prawns that had been in darkness or in light for 14 days. Supraesophageal ganglia were removed from prawns that had been collected less than 24 hours prior to use in the experiment and triturated with sufficient sea water to yield extracts that contained $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ and 1 organ per 0.02 ml. sea water. Each extract was assayed on 10 one-eyed prawns in black pans under an illumination of 31 ft. c. The responses were observed for three hours. This experiment was performed three times. The data of the three experiments were then averaged and the means were used in the preparation of Figure 2.

Two facts were apparent from inspection of the figure: (1) the higher the concentration of supraesophageal ganglia in the extracts, the greater the distal pigment index 30 minutes after the extracts were injected, and (2) the rate of return of the index to the control level was faster with the higher concentrations than with the lower ones. Furthermore, no indication of a dark-adapting response was apparent even at the highest concentration.

As a measure of the light-adapting potency of the extracts the differences between the means of the distal pigment indices of the controls and of prawns receiving extracts of supraesophageal ganglia were summed. The sums (potency values) were used to compile Figure 3. In like manner, potency values were calculated for the extracts of the supraesophageal ganglia of the specimens that had been in light (0.034) and in darkness (0.204) for 14 days. The coordinates for the supraesophageal ganglia of specimens that had been in light 14 days (0.2 organ/0.02 ml. and a potency value of 0.034) almost fell on the curve of Figure 3. As the curve was drawn, the potency value expected from an extract containing two-tenths of the supraesophageal ganglia of one prawn per 0.02 ml. was 0.045. Illumination, therefore, had no appreciable effect on the amount of light-adapting hormone. Further inspection of the figure revealed that a potency value of 0.204 would be caused by an extract of supraesophageal ganglia of freshly collected prawns with a concentration of eight-tenths of an organ per 0.02 ml. Presumably, therefore, the amount of light-adapting hormone in the supraesophageal ganglia quadrupled during the two weeks the specimens had been in the darkroom.

Effect of time in darkness on the ability of the distal retinal pigment to respond to a dark-to-light change

The object of this experiment was to determine if the period of time specimens were kept in darkness influenced their subsequent rate of light-adaptation. Prawns were collected, put into white enameled pans and placed in darkness. Two hours later the prawns were exposed to an illumination of 31 ft. c. for 30 minutes. The distal pigment indices of 10 specimens were determined and the prawns were again put in darkness. They were subsequently exposed to illumination after having been in darkness 3, 5, 7, 10, 12 and 14 days. The mean distal pigment indices determined after 30 minutes of illumination gradually increased.

The experiment was repeated and the results were substantially the same. The data of the two experiments were averaged and the means were used in the preparation of Figure 4. The number of prawns gradually decreased while each experiment was in progress. Totals for both experiments were: 20 on the first

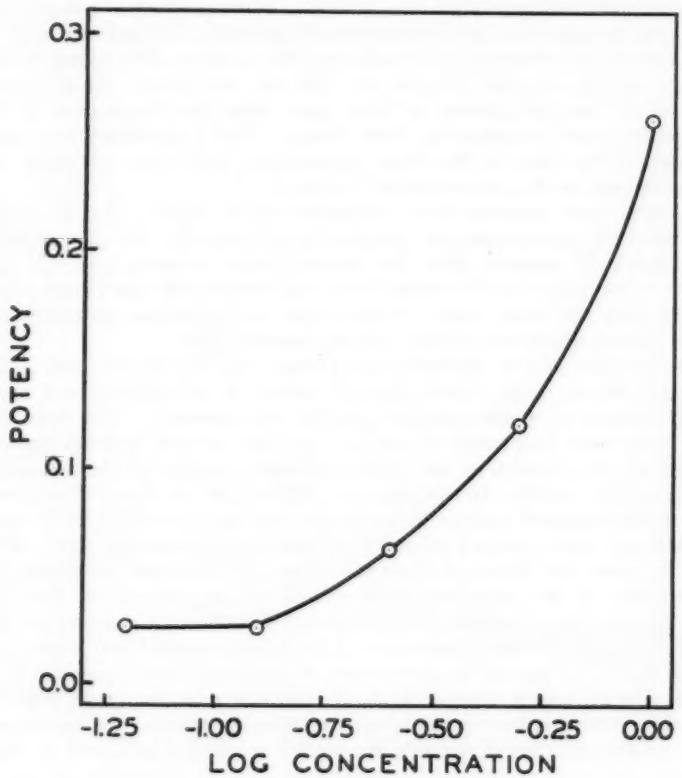

FIGURE 3. Relationship between light-adapting potency and concentration of extracts of supraesophageal ganglia of specimens collected less than 24 hours before use. The concentration is the logarithm of the number of supraesophageal ganglia each assay animal received triturated in 0.02 ml. sea water.

and third days, 16 on the fifth day, 15 on seventh and tenth days, 12 on the twelfth day and nine on the last day of the experiment.

The indices recorded on the first and last days of both experiments were analyzed statistically (Table I, Analysis 6); the difference between the means was highly significant. The longer the prawns had been in darkness, the faster the distal pigment approached the fully light-adapted position.

Digestion of the light-adapting and dark-adapting hormones by trypsin

The object of the first experiment of this series was to demonstrate that a boiled solution of trypsin would not inactivate the retinal pigment hormones. After boiling, the enzyme would have been inactivated but some contaminant might have possibly destroyed the hormones. This possibility had to be ruled out. For the first experiment of this group a 2% solution of trypsin in sea water was prepared; at the same time a freshly prepared extract of eight eyestalks in 0.2 ml. sea water was boiled for 30 seconds and centrifuged. Equal volumes of the boiled trypsin solution and the eyestalk extract were mixed together. The

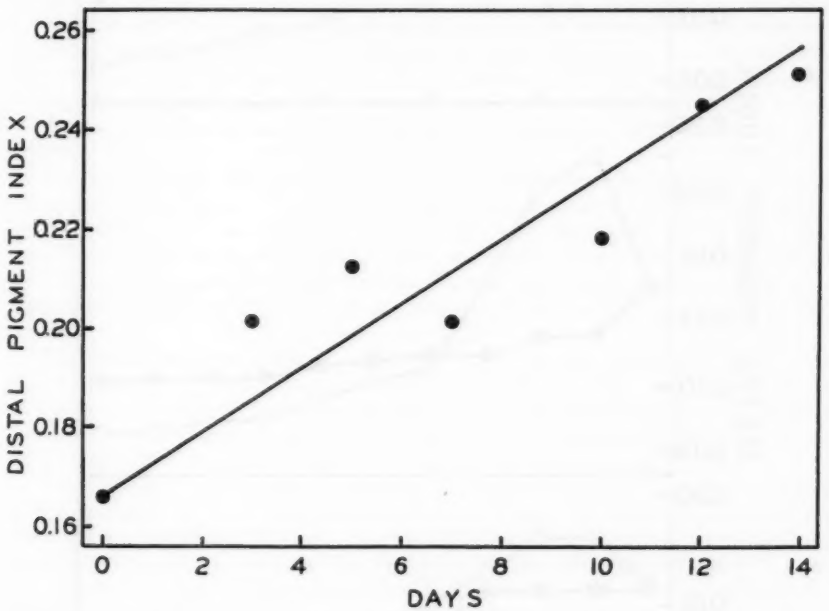

FIGURE 4. The distal retinal pigment index 30 minutes after prawns had been taken from darkness and placed on a white background under an illumination of 31 ft. c., versus the number of days the specimens had been in darkness.

mixture was then injected into one-eyed prawns in black pans under an illumination of 22 ft. c. Boiled trypsin solution diluted with an equal volume of sea water was injected into the controls.

The results were those that were anticipated. A light-adaptational response occurred and lasted two hours. A dark-adaptational response followed. The extent of the latter was not followed to its completion. Fingerman, Lowe and Sundararaj (1959) reported that the dark-adaptational response lasted at least five hours. The experiment was performed two more times and the results were

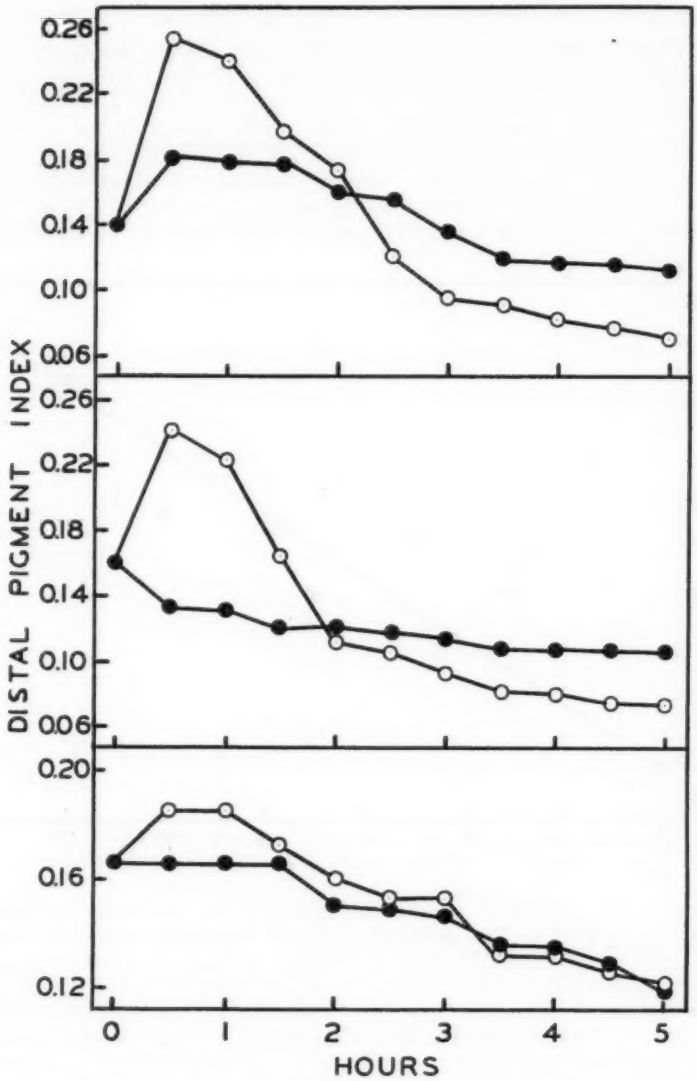

FIGURE 5. Responses of the distal retinal pigment to extracts of eyestalks. Top, the extract was prepared in boiled, centrifuged trypsin solution, circles. Control of boiled, centrifuged trypsin in sea water, dots. Middle, extract prepared in sea water and kept for one hour, circles. Sea water control, dots. Bottom, extract in trypsin solution for one hour, circles. Control, dots.

the same. The data of the three experiments were averaged and the means were used in the preparation of the top portion of Figure 5.

These data were analyzed statistically (Table I, Analyses 7, 8) and the amounts of light-adaptation and dark-adaptation were significant. The distal pigment indices obtained 30 and 60 minutes after the extracts were injected were used for Analysis 7; the indices determined 3.0, 3.5, 4.0 and 4.5 hours after the extracts were injected were used in Analysis 8.

For the next experiment an extract was prepared of 18 eyestalks in 0.45 ml. sea water. Four-tenths ml. of the centrifuged extract was divided into two equal portions. To one fraction was added 0.2 ml. sea water and to the other 0.2 ml. of 2% trypsin solution in sea water. Both tubes were kept for one hour at room temperature, 24° C., at which time the material in the tubes was boiled for 30 seconds and centrifuged. The supernatant from both tubes was injected into assay animals. Boiled, centrifuged trypsin solution was injected into one-eyed prawns as a control for the extract of eyestalks treated with trypsin. As a control for the extract not treated with trypsin, sea water was injected into one-eyed prawns.

The extract that did not contain trypsin caused light-adaptation and subsequent dark adaptation. The trypsin-treated extract caused a small amount of light-adaptation and no dark-adaptation. The experiment was performed two more times and each time the results were essentially the same. The data of the three experiments were averaged and the means were used in the preparation of the middle (pure extract) and bottom (trypsin-treated extract) portions of Figure 5.

Statistical analyses (Table I, Analyses 9, 10) of the results depicted in the middle portion of Figure 5 showed that the light-adapting and dark-adapting effects were significant. The distal pigment indices determined 30 and 60 minutes after the extracts were administered were used for Analysis 9, the indices obtained after 4.0, 4.5 and 5.0 hours for Analysis 10.

To determine if the difference between the degrees of light-adaptation produced by the pure and trypsin-treated extracts was significant, the distal pigment indices determined 30 and 60 minutes after injection of the extracts were analyzed statistically (Table I, Analysis 11) and found to be significant at the 1% level. The amount of light-adapting hormone in the extract had been reduced by the trypsin.

Electrophoretic analysis of the retinal pigment hormones

The object of the last set of experiments was to separate the dark-adapting hormone in the eyestalk from the light-adapting one. Separation was accomplished by filter paper electrophoresis performed in the manner described above. The experiment was performed three times.

The results of the three experiments were qualitatively alike and were averaged (Fig. 6). The region of application of the extract to the strip and the three-inch section of the strip on the cathodal side of the origin contained light-adapting and dark-adapting substances. Fluid obtained from the three-inch section on the anodal side of the origin produced dark-adaptation alone. Distal pigment indices determined 90, 120 and 150 minutes after the extracts from the anodal portion

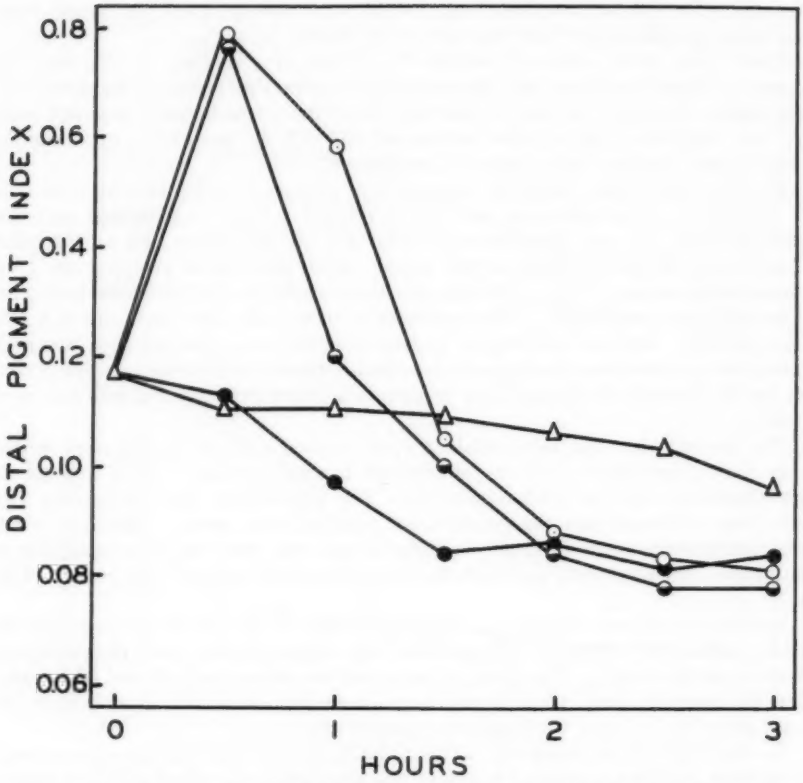

FIGURE 6. Responses of the distal retinal pigment to extracts of eyestalks. These extracts had been subjected to filter paper electrophoresis. Circles, fraction that migrated toward the cathode; dots, fraction that migrated toward the anode; half-filled circles, fraction that remained at the origin; triangles, control.

of the strips and the control strips had been injected were compared statistically (Table I, Analysis 12). The amount of dark-adaptation was highly significant.

DISCUSSION

The amount of light-adapting hormone in the supraesophageal ganglia increased in specimens of *Palaemonetes* kept in darkness. This hormone in the supraesophageal ganglia, therefore, must be normally involved in regulation of the distal retinal pigment. The site of release of the light-adapting hormone from the supraesophageal ganglia is unknown. It could be transported via axons to a storage and release center in the eyestalks (*e.g.* sinus gland) or could be released directly from neurosecretory cells in the supraesophageal ganglia into the blood.

The light-adaptational response produced by the strongest extract of supraesophageal ganglia (Fig. 2) was of the same magnitude as that produced by the extracts of eyestalks (Fig. 5). However, the extracts of the supraesophageal ganglia did not cause a dark-adaptational response. The dark-adaptational response produced by the eyestalk extracts might have been considered overcompensation by the prawns in removing the injected light-adapting hormone from the blood. This interpretation, however, can not be correct in view of the results obtained with the extracts of the supraesophageal ganglia (Fig. 2).

The data shown in Figure 4 reveal that the prawns adapted faster to light the longer they had been in darkness. The observation that the supraesophageal ganglia of prawns kept in a darkroom for 14 days contained more light-adapting hormone than the organs of illuminated specimens can explain the results shown in Figure 4. The animals light-adapted at a faster rate with increased time in darkness because they had a greater store of light-adapting hormone to use.

Some physico-chemical properties of the retinal pigment hormones of *Palaemonetes* can be adduced from these experiments. Both hormones are heat-stable. With regard to the chemical nature of the retinal pigment hormones, there is inconclusive evidence that the neurosecretory products of crustaceans are polypeptides. The sensitivity of these substances to trypsin and chymotrypsin suggests the presence of peptide bonds. Pérez-González (1957) found that the hormone that dispersed the black pigment in the fiddler crab, *Uca pugilator*, was inactivated by chymotrypsin. Kleinholz (personal communication) found that the light-adapting substance in the eyestalk of *Palaemon serratus* at Naples, Italy, was sensitive to trypsin and chymotrypsin. Evidence was presented in Figure 5 for the trypsin sensitivity of the retinal pigment dark-adapting and light-adapting hormones in *Palaemonetes*. Trypsin and chymotrypsin, however, also have weak esterase activity and inactivation by these enzymes, alone, is not sufficient proof of the peptide nature of these neurosecretory substances.

Both retinal pigment hormones migrated in an electric field (Fig. 6). At pH 9.0 the dark-adapting hormone separated from the light-adapting hormone so that light-adaptation did not precede the dark-adaptational response. In contrast, whenever extracts of eyestalks were injected before electrophoresis a light-adaptational response occurred before the dark-adaptational one (top portion of Figure 5). Because the dark-adapting hormone was found at the origin and on both sides of it, pH 9.0 must be close to its isoelectric point. The isoelectric point of the light-adapting hormone must be higher than pH 9.0 because no light-adapting hormone was found on the anodal side of the origin.

Summary and Conclusions

1. Some physico-chemical properties of the hormones activating the distal retinal pigment of the prawn *Palaemonetes* were determined.

2. The light-adapting and dark-adapting hormones were heat-stable, inactivated by trypsin and migrated in an electric field. These findings suggest that both hormones are polypeptides.

3. The amount of light-adapting hormone increased four-fold in the supraesophageal ganglia of prawns kept in darkness 14 days. The quantity of this hormone in the supraesophageal ganglia of specimens illuminated for 14 days did

not change detectably. These results demonstrated that the light-adapting hormone from the supraesophageal ganglia was used by the prawns in regulating the distal retinal pigment.

4. The longer prawns were kept in darkness, the faster was the rate of migration of the distal pigment toward the light-adapted position when prawns were illuminated. This increase was explained by the increase in the light-adapting hormone in the supraesophageal ganglia of specimens kept in darkness.

5. The dark-adapting hormone was separated from the light-adapting one by filter paper electrophoresis at pH 9.0.

LITERATURE CITED

ALLEE, W. C., 1923. The distribution of common littoral invertebrates of the Woods Hole Region. Studies in Marine Ecology I. *Biol. Bull.*, **44**: 167–191.

BROWN, F. A., JR., M. N. HINES AND M. FINGERMAN, 1952. Hormonal regulation of the distal retinal pigment of *Palaemonetes*. *Biol. Bull.*, **102**: 212–225.

FINGERMAN, M., M. E. LOWE AND B. I. SUNDARARAJ, 1959. Dark-adapting and light-adapting hormones controlling the distal retinal pigment of the prawn *Palaemonetes vulgaris*. *Biol. Bull.*, **116**: 30–36.

HOLTHUIS, L. B., 1952. A general revision of the Palaemonidae (Crustacea Decapoda Natantia) of the Americas. II. The subfamily Palaemoninae. *Occasional Papers; Allan Hancock Foundation Publ.* Number 12, pp. 1–396.

KLEINHOLZ, L. H., 1936. Crustacean eye-stalk hormone and retinal pigment migration. *Biol. Bull.*, **70**: 159–184.

KLEINHOLZ, L. H., AND F. G. W. KNOWLES, 1938. Studies in the pigmentary system of Crustacea. III. Light-intensity and the position of the distal retinal pigment in *Leander adspersus*. *Biol. Bull.*, **75**: 266–273.

PÉREZ-GONZÁLEZ, M. D., 1957. Evidence for hormone-containing granules in the sinus glands of the fiddler crab, *Uca pugilator*. *Biol. Bull.*, **113**: 426–441.

SANDEEN, M. I., AND F. A. BROWN, JR., 1952. Responses of the distal retinal pigment of *Palaemonetes* to illumination. *Physiol. Zoöl.*, **25**: 223–230.

SEX-LINKED INHERITANCE IN ULVA

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In an earlier report (Föyn, 1959) mention was made of two autosomal mutants, Slender (*S1*) and long (*1*), both of which spontaneously arose in cultures of *Ulva mutabilis* and both of which exhibit sex-controlled manifestation. Found among the young gametophytes from a crossing between the two mutants was a small dwarfed individual entirely different from the types hitherto known. The individual was isolated and proved to belong to the − -sex. In order to determine whether it was a matter of a new hereditary type or only a phenotypical abnormality, the gametes were put to parthenogenesis. The new germ plants were all of the same kind as the mother plant, and thus probably represented a new mutant. As described earlier (Föyn, 1958) parthenogenesis in this species is normally connected with doubling of the chromosome number. The parthenogenetic plants therefore became diploid and produced (haploid) zoöspores. Figure 1 shows a few stages in their development towards gametophytes.

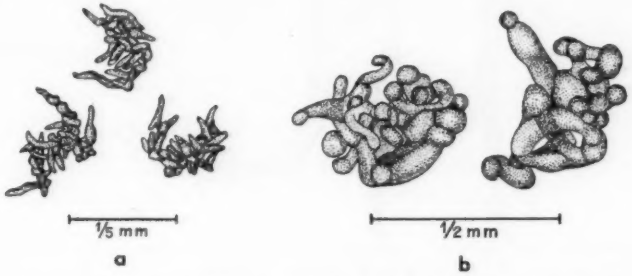

FIGURE 1. *Ulva mutabilis.* Young *dw S1* individuals from zoöspores. a, 10 days old; b, 17 days old.

The gametophytes were fertile after a good month and they were then crossed with the wild-type. The zygotes germinated immediately to germ plants of the sporophyte generation. As Figure 2 shows, they resemble the sporophyte germ plants after crossing between the Slender-type and the wild-type but diverge from them by the tapered top and the weakly developed rhizoids which do not grow out from the entire basal part, but only from a single point medially below this. With some germ plants the basal part can also grow one or a few short branches such as in the individual on the right of the figure, but this is rare.

The hybrid sporophytes when about 2½ months old formed zöospores. The plants had then grown to a length of 25–30 cm., flat, and with even width (ca. 1 cm.) band. Zoöspores from one of the plants were transferred to a petri dish

which was thereupon placed in darkness until the following day. The zoöspores had by then become distributed evenly over the bottom of the dish and had affixed themselves to the glass. Inspection 10 days later found the bottom over-grown by various types of germ plants. A precise count was taken within a circle-shaped area of 2.3 cm. diameter, and the various types were at the same time separated, each plant being loosened from the under-bedding with the aid of a glass needle and transferred to a new petri dish. Altogether, within the limited area 728 germ plants were found. Of these, 189 belonged to the wild-

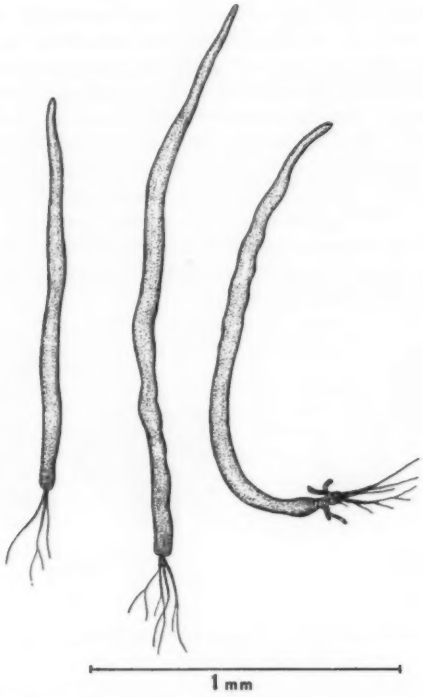

FIGURE 2. *Ulva mutabilis.* Twelve-day-old sporophytes from a cross between a *dw S1* gametophyte and a gametophyte of the wild-type.

type and 138 to the Slender-type. The remaining 401 consisted for the greater part of germ plants of the same appearance as in Figure 1a and thus resembled the pristine mutant plant completely. A smaller number were more simple, built more clumpily, and were only weakly ramified (Fig. 3a). Every transition between the two types was found, however, and an assortment into two distinct groups had, therefore, to be abandoned. All of them were reckoned as belonging to the mutant type. Thirty-six germ plants could not be identified; they were, partly, of different shape and dissimilar to the types hitherto known. The plants

were accordingly carried forward to sex-maturity and the gametes were put to parthenogenetic development. A survey of the parthenogamete plants 14 days later showed that not one of them represented new mutants. Four belonged to the wild-type, 21 to the Slender-type and 11 to the mutant-type. The final result of the count was therefore:

wild-type:	193 individuals (26.51%)
mutant-type:	376 individuals (51.65%)
Slender-type:	159 individuals (21.84%)

It is therefore a matter of a crossing with two independently segregating factors of which the one is *S1*. The other has been designated *dw*. Half of the 376 germ plants of the mutant type must have both the chromosomes with *S1* and the chromosome with *dw* the other half, only the chromosome with *dw*.

Three dishes with altogether 220 mutant germ plants were placed under the source of light and, gradually, as the plants grew, the largest were isolated, carried forward to sex-maturity, and crossed with the wild-type. It soon became

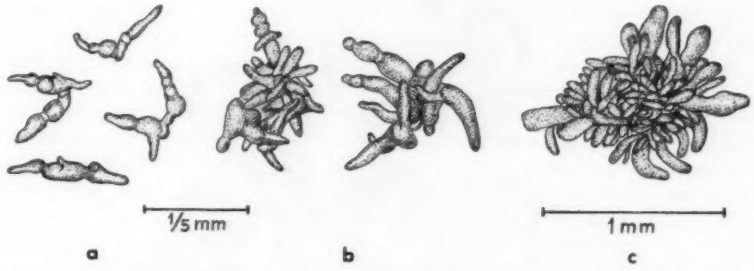

FIGURE 3. *Ulva mutabilis*. Young *dw* individuals from zoöspores. a, 12 days old; b, 19 days old; c, 30 days old.

evident that *dw S1* individuals grow much more rapidly and, therefore, become fertile earlier than *dw*-individuals. The first crossings all resulted in sporophytes of the same kind as the mother sporophyte (Fig. 2). It was only after 84 plants had been tested that the crossings between the three successive individuals and the wild-type gave another result, namely, sporophytes of the wild-type. Upon the formation of zoöspores eleven weeks later, they segregated into the mutant and the wild-type in the ratio 1 : 1. A precise count was not taken as already at the first glance into the microscope it was evident that the two types were represented equally strongly.

For each crossing undertaken, the gametes of the mutant partner were put to parthenogenesis. In the first 84 cases mentioned the gametes developed, as expected, mainly to germ plants of the ramified type, but also a few developed to individuals of the simple type. After the three crossings following, however, the ratio was reversed: most of the parthenogamete plants became simple whereas only a few became considerably ramified. Even if weak or no ramification accordingly must be said to be a typical character of the pure *dw* germ plant,

it is no certain distinguishing feature. One of the three above-mentioned dishes having mutant germ plants contained only individuals of the simple type, altogether 20; 13 of them later yielded gametes, 10 proved to be pure *dw* plants and the remaining three, *dw S1* individuals. The two other dishes each contained 100 germ plants, chosen casually, without regard to type or size. Of these, altogether 197 have been tested in crossing with the wild-type with the following result: 118 were *dw S1* individuals, 79, *dw* individuals.

It is apparent from Figures 3c and 4 that the *dw* plants also later become considerably ramified, frequently much more so than *dw S1* plants. The fertile *dw* individual looks like a rosette of young wild-type plants grown together. The thallus is dark green, often folded, and gives a stunted impression. The two layers of cells are either grown together throughout or are separated from each other along the border of the thallus. The thickness of the layers is greater than

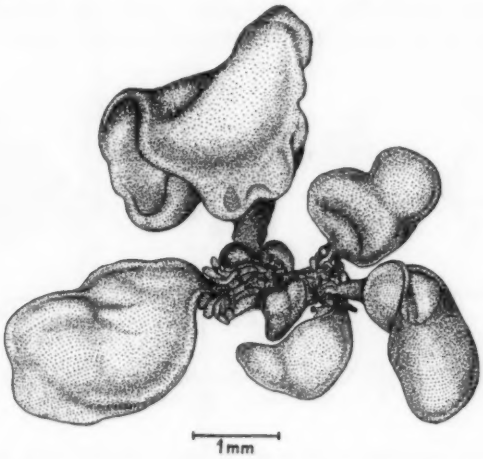

FIGURE 4. *Ulva mutabilis*. A full grown and fertile *dw* sporophyte. Three months old.

in the wild-type. The rhizoids are weakly developed or are not at all distinguishable. It is because of this dwarf-like appearance that the mutant factor has been termed *dw*.

The fertile *dw S1* plants much resemble the *dw* plants. Nor do they become larger than these but the two layers of cells are often free throughout and, partially, distend strongly from each other. Most frequently, they have the usual green colour.

More interesting than these results, however, was that all the 210 mutant individuals (both *dw* and *dw S1* individuals) which had been carried forward to sex maturity proved to belong to the − -sex. Investigation was therefore made as to how the 193 individuals of the wild-type and the 159 Slender individuals behaved in this respect. Where the latter are concerned, it had already been

observed during the counting that most of them were ramified which, with this mutant, is a quite certain sign that they are + -individuals (see below). Only 61 were unramified and these were therefore isolated and carried forward to sex maturity. All of them revealed themselves as + -plants. Of the wild-type, 100 individuals were tested with regard to sex, all with the same result, namely, that they belonged to the + -sex. Also in different later crossings which, in another connection, have been performed with *dw*-plants, individuals with the *dw* factor have proved to be of the − -sex. The factor *dw* must therefore be located in the same chromosome as the factor(s) for the − -sex.

It has not been possible to establish cases of crossing-over between *dw* and the sex-factor(s). The crossing best suitable for the discovery of such cases is that between *dw S1* and *dw*$^+$ *S1* individuals. The crossing can naturally only result in the same types of gametophytes as the parents, and, apart from eventual cross-overs, the *dw S1* plants will belong to the − -sex and the *dw*$^+$ *S1* plants, the + -sex. As described earlier (Föyn, 1959) the germ plants of the − -sex of the Slender race are always long, slim and unramified, whereas the germ plants of the opposite sex can either be like the − -individuals or be small and considerably ramified. This variation rests upon hitherto unknown environmental conditions, as + -zoöspores from one and the same sporophyte after one swarming can develop to germ plants, all of which are small and ramified, and, after another, to both ramified and unramified, or to unramified germ plants only. In the cases where the first possibility is realized from the + -zoöspores of *dw dw*$^+$ *S1 S1*-sporophyte, naturally a − -individual of the Slender-type will immediately become evident. Of the 14 petri dishes with their bottom wholly covered by haploid germ plants from two *dw dw*$^+$ *S1 S1*-sporophytes, three contained exclusively small and ramified germ plants. In the one dish the germ plants originated from the one, and, in the other two, from the second sporophyte. In two of the dishes there were more than 4000 individuals, in the third, more than 5000 individuals; thus, from among more than 6500 *dw*$^+$ *S1*-individuals, not a single cross-over was observed.

The author would like to express his thanks to Miss Ingeborg Gjöen for the execution of the drawings, and to Mr. John C. Aird for the translation of the manuscript.

Summary

A recessive sex-linked mutant is described that arose spontaneously in cultures of *Ulva mutabilis*. It has not been possible to establish cases of crossing-over between the sex chromosomes.

LITERATURE CITED

Föyn, B., 1958. Über die Sexualität und den Generationswechsel von *Ulva mutabilis* (N.S.) *Arch. f. Protistenkund.*, **102**: 473–480.

Föyn, B., 1959. Geschlechtskontrollierte Vererbung bei der marinen Grünlage *Ulva mutabilis*. *Arch. f. Protistenkund.*, **104**: 236–253.

INFLUENCE OF CARBONIC ANHYDRASE INHIBITORS ON SHELL GROWTH OF A FRESH-WATER SNAIL, PHYSA HETEROSTROPHA [1]

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The enzyme carbonic anhydrase catalyses reversibly the reaction

$$CO_2 + H_2O \rightarrow H_2CO_3$$

(Berliner and Orloff, 1956) and has been shown to accelerate both deposition and dissolution of calcium carbonate *in vitro* (Meldrum and Roughton, 1933). In view of its possible implication in shell formation, the mantle tissues of molluscs have been examined and the enzyme demonstrated in several of them (Florkin and de Marchin, 1941; Maetz, 1946; Freeman and Wilbur, 1948; Wilbur and Anderson, 1950; Stolkowski, 1951; Tsujii and Machida, 1953; Ikinago, 1954; Kawai, 1954a, 1954b; Larraneta and Ponz, 1954; Clark, 1957). However, among 20 species examined, Freeman and Wilbur (1948) found little or no carbonic anhydrase activity in mantle tissue in two, a pelecypod and a gastropod, both shell-formers, and Stolkowski (1951) found none in the mantles of *Ostrea edulis* and several other shell-forming molluscs. Such results suggest that at least in some species the enzyme is not essential for shell formation.

Stolkowski (1951) apparently first used a carbonic anhydrase inhibitor, benzenesulfonamide, in studies of the role of the enzyme in molluscs, observing decreased shell regeneration in *Helix aspersa* in the presence of the drug. He concluded that the enzyme is important in determining the crystalline form in which calcium carbonate appears in the shell. Wilbur and Jodrey (1955) found that two carbonic anhydrase inhibitors, 2-benzothiazolesulfonamide and Diamox (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), markedly decrease the rate of deposit of Ca^{45} in the shell of *Crassostrea virginica*. Abolins-Krogis (1958) in a study of shell regeneration in *Helix pomatia* concludes on the basis of as yet unpublished data on carbonic anhydrase inhibition that (p. 36) the enzyme "is necessary for the formation of calcium carbonate crystals in the regenerating shell." These studies suggest a role of carbonic anhydrase in shell formation but are inconclusive in establishing it. The two inhibitors used by Wilbur and Jodrey, 2-benzothiazolesulfonamide and Diamox, exhibited quite different extents of inhibition of Ca^{45} deposition in the intact animal, only Diamox affecting deposition in a manner consistent with the hypothesis that the results were due to carbonic anhydrase inhibition. Even though in the mantle-shell preparation used in part of the studies the two drugs had effects consistent with the idea that the enzyme is important in determining the rate of calcium deposit, the side effects of one of the drugs suggest caution in interpretation of the data. Further, the

[1] This study has been supported in part by a grant from the Southern Fellowships Fund.

data are consistent with another interpretation, that carbonic anhydrase influences the exchange of calcium between the shell and the extrapallial fluid. This is an exchange which may not include the reactions which serve as a pathway for shell formation.

The study of Abolins-Krogis (1958) indicates that calcium carbonate crystals did not develop in the presence of a carbonic anhydrase inhibitor. Since the reaction catalyzed by the enzyme occurs at a very appreciable rate, proceeding 90 per cent to equilibrium in 200 seconds (Berliner and Orloff, 1956), it would be expected that during inhibition of the enzyme deposition of calcium carbonate would occur at a decreased rate but would not cease.

One method of resolving the dilemma would be to study the growth of shell in the presence of a number of carbonic anhydrase inhibitors, for if a series of side effects were operative among the drugs in decreasing shell development these effects should show different extents or at least statistically modify in different ways the pattern for the growth of the populations treated with different drugs. On the other hand, if the effect on shell development is statistically similar in the case of several known carbonic anhydrase inhibitors this would strengthen the evidence for a direct role of the enzyme in shell development. In the present study the hypothesis that carbonic anhydrase is important in molluscan shell development has been tested by a study of the influence of five carbonic anhydrase inhibitors on the growth of the shell of the pond snail, *Physa heterostropha*.[2]

Methods

Adult snails of a local population were maintained as stock cultures on lettuce with oyster shell chips in the bottom of aquaria. Young snails not over a month old were removed from the stock aquaria as required and placed in 4-inch fingerbowls containing 100 ml. water which had been equilibrated with precipitated calcium carbonate for at least a week. Three or four snails from about 2 to 5 mm. in length, which were individually distinguished in the course of measurements, were placed in each fingerbowl along with lettuce. Twenty-four hours later each snail was measured, using a micrometer calibrated to 0.01 mm., and the water was replaced. At this time appropriate volumes of solutions of the drugs (made up in the $CaCO_3$-saturated water) were substituted for a part of the water in dishes containing the experimental animals. Subsequently the water or inhibitor solution was replaced daily and lettuce was added as needed. (For information on feeding, see Table I.) The snails were measured on alternate days for ten days. In the few cases in which snails were lost, died or were accidentally killed, the measurements were included if data were available for six or more days of growth. (Such losses were: 8 snails died during the major series of observations, 6 were killed accidentally during handling, and there were 3 losses which were unaccounted for among 672 snails.)

The experiment was carried out in the room in which the snails were reared, at a temperature which in most experiments approximated 26° C. (In a preliminary study the temperature approximated 21°, as indicated in appropriate places in the tables.)

[2] The author appreciates the identification of the snail by Dr. H. Van der Schalie of the University of Michigan.

TABLE I

Growth of Physa heterostropha fed in three ways. Data for snails having median lengths during growth periods of 3.00–5.99 mm.

Feeding	No. snails	Growth (mm./day)		
		Median	Mean	Range
I	12	0.11	0.10 ± 0.015	0.05–0.13
II	55	0.25	0.27 ± 0.010	0.07–0.38
III	30	0.40	0.41 ± 0.014	0.28–0.49

Observations were at *ca.* 26°. Fresh lettuce was available to all snails continuously, the amount of decaying lettuce varying. In feeding method I, each morning any piece of lettuce showing signs of decay was removed and replaced with fresh lettuce. In method II, each piece of lettuce was left until all parts showed signs of decay. Then it was removed and replaced with fresh lettuce. In method III, decaying lettuce was available at all times and decaying pieces were removed when only plant fibers remained.

The inhibitors used were Diamox (2A),[3] 2-benzothiazolesulfanilamide (2B)[3] (Miller, Dessert and Roblin, 1950), benzenesulfonamide (BS), p-toluenesulfonamide (TS) (Krebs, 1948) and sulfanilamide (SA) (Mann and Keilin, 1940).

RESULTS

Examination of preliminary data in graphs indicated approximately uniform growth rates for snails having median lengths ranging from 2 to 6 mm. during growth periods and lower rates for those having lengths outside this range. More critical examination of the data and subsequently of data for all control groups, using the Kruskal-Wallis test (Kruskal and Wallis, 1952), indicates the range of lengths having statistically uniform growth rates is more restricted, from 3.00 to 5.99 mm. when data are examined in classes having 0.50 mm. ranges. All data for controls have been examined and, with the exception of a single class among those subjected to feeding method I, show no statistically significant variations in growth rates among classes having median lengths of 3.00 to 5.99 mm. within any method of feeding at a given temperature. As determined by the Kruskal-Wallis test, probability (p) for controls ranges from 0.3 to 0.8, with the exception indicated. Statistically significant variations among classes within experiments were obtained in each case in which a broader range of median lengths was used. Inclusion of all data in calculations would have made no difference in the over-all picture, for among the control experiments the mean growth rates of snails subjected to different feeding regimes would have been 0.10, 0.24 and 0.37 instead of 0.10, 0.25 and 0.41, and similar differences were found for experimental populations. The difference is in part determined by the proportion of measurement made on snails outside the range 3.00 to 5.99 mm.

Preliminary experiments indicated that the manner of feeding greatly influenced the rate of growth. Although fresh lettuce supported slow growth, the most rapid growth was obtained only if decaying lettuce was available to the

[3] Diamox and 2-benzothiazolesulfanilamide were supplied by Dr. Emmanuel Waletzky of American Cyanamid Co.

TABLE II

Growth of Physa heterostropha *in 2-benzothiazolesulfonamide. (Data for periods in which median lengths were 3.00–5.99 mm.)*

Feeding	Temp. (°C.)	Conc. 2B (mg./l.)	No. snails	Growth (mm./day)		
				Median	Mean	Range
I	21	0.0	12	0.11	0.10 ± 0.015	0.05–0.13
		0.1	12	0.12	0.11 ± 0.020	0.08–0.19
		0.5	12	0.10	0.12 ± 0.018	0.01–0.28
		1.0	12	0.01	0.02 ± 0.005	0.00–0.08
II	26	0.0	55	0.25	0.27 ± 0.010	0.07–0.38
		0.1	17	0.21	0.22 ± 0.013	0.12–0.30
		0.2	15	0.17	0.18 ± 0.013	0.06–0.26
		0.5	49	0.05	0.07 ± 0.005	0.00–0.14
		1.0	14	0.00	0.00 ± 0.001	0.00–0.02

snails at all times. The rate of growth of snails used in control experiments at 26° has been compared for three feeding methods with the results indicated in Table I.

In all experiments subsequently presented in this report feeding was by method III which gave most rapid growth, unless otherwise indicated, and data are for growth periods during which the median lengths of the snails were from 3.00 to 5.99 mm.

The effect of 2B on growth was quite different from that of the other four drugs and so is presented separately in Table II. Additional information on the effects of 2B were obtained in preliminary experiments conducted at *ca.* 21°. In these experiments, of 27 snails exposed to concentrations of 2B from 2.0 to 10.0

TABLE III

Comparison of growth rates of Physa heterostropha *treated with concentrations of 2A, TS, BS and SA showing maximum inhibition of growth with untreated controls. Data for periods in which median lengths of snails were within the range 3.00–5.99 mm.*

Feeding	Temp.* (°C.)	Drug	Drug conc. (mg./l.)	No. snails	Mean growth (mm./day)	Per cent inhibition	p**
III	21	none	0.0	16	0.23 ± 0.014		
		2A	0.5–15.0	25	0.15 ± 0.009	35	<0.00003
II	26	none	0.0	55	0.27 ± 0.010		
		2A	2.0–5.0	65	0.15 ± 0.007	44	0.0013
III	26	none	0.0	30	0.41 ± 0.014		
		BS	50	15	0.26 ± 0.014	37	
		TS	50	16	0.26 ± 0.016	37	<0.00003
		SA	10.0–50	30	0.28 ± 0.015	32	

* Temperatures varied within approximately two degree ranges.

** Values for p have been determined using the Mann-Whitney U test for comparing the data for control and experimental groups (Mann and Whitney, 1947).

mg. per liter, 24 died within two days and the remaining three within four days. At concentrations of 0.5 and 1.0 mg. per liter, *ca.* 50% mortality occurred within four days. One of six died between the tenth and twelfth day in 0.25 PPM 2B and none of 15 maintained as controls or in 0.05 PPM 2B died within 12 days. Mortality was lower at 26°, 4 of 87 snails dying within 10 days in 2B solutions of concentrations 0.5 and 1.0 mg. per liter. Among 55 controls and 32 snails in 0.1 or 0.2 PPM 2B, none died within 10 days.

The four remaining drugs when used with snails growing at the more rapid rates (feeding methods II and III) showed maximum inhibition of growth to comparable extents. The results of these experiments are presented in Table III. The data for concentrations showing maximum inhibition have been examined in classes (as indicated above for control experiments) using the Kruskal-Wallis test and found to be statistically homogeneous (p ranges from 0.1 to 0.5). Data for BS, TS and SA obtained in simultaneous observations have been similarly compared as a group with their controls. The value of p thus obtained was < 0.00003.

A series of experiments using Diamox was run to compare inhibition of growth of slowly growing snails with that of rapidly growing ones. Mean growth rates

TABLE IV

Growth rates of snails subjected to various concentrations of Diamox and fed for slow growth (Feeding I) at 26° C.

Conc. Diamox	0.0	0.1	0.2	0.3	0.4	0.5	1.0	1.5
Mean growth rate (mm./day)	0.10	0.12	0.10	0.07	0.10	0.08	0.10	0.10
S.E. of mean	0.015	0.019	0.016	0.013	0.016	0.011	0.014	0.016

observed are shown in Table IV. When these control and experimental groups are compared, no statistically significant differences between the growth rates of the groups of snails are found ($p = 0.3$, using the Kruskal-Wallis test).

DISCUSSION

The effect of 2B on the snail *Physa heterostropha* is quite different from that of the other drugs studied and is of a nature which indicates its action on the snail is not simply one of inhibition of carbonic anhydrase. This result agrees well with the conclusion of Wilbur and Jodrey (1955) that this drug has an effect on calcium deposition in the oyster that is independent of its inhibition of carbonic anhydrase and in their experiments was shown to be independent of the mantle.

The results of experiments using Diamox, BS, TS, and SA with snails fed for rapid growth are consistent with the hypothesis that rapid shell growth in this mollusc depends upon the activity of carbonic anhydrase and it is unlikely that such congruence of effects would occur if the four drugs were acting on different enzyme systems. At low growth rates induced by feeding method I, Diamox did not further decrease growth rates, indicating that under at least certain circumstances of slow growth carbonic anhydrase activity is insignificant in shell growth

of the snail. Such a result does not, however, preclude the possibility that the enzyme may have increased importance in determining the rate of growth under other circumstances limiting shell development, as low temperatures.

The results here reported are entirely consistent with the hypothesis that under certain circumstances carbonic anhydrase is essential for rapid shell development in the snail used but that in some conditions the enzyme is not necessary for shell growth.

Summary

1. The rate of growth of *Physa heterostropha* may be controlled in the laboratory by using different modes of feeding.

2. Under the conditions of the experiments, growth rates for each feeding method, with the possible exception of that giving slowest growth, are constant over the range 3.00 to 5.99 mm. median shell lengths during two-day growth periods but are slower at both higher and lower lengths.

3. The drug 2-benzothiazolesulfonamide completely inhibits growth of the snail at concentrations about 1 mg. per liter. At 21° C. it is lethal at concentrations above this but appears to be somewhat less toxic at 26° C. Its effect is not due to the action of the drug as a carbonic anhydrase inhibitor but to an unknown side effect.

4. Diamox, benzenesulfanilamide, p-toluenesulfonamide and sulfanilamide, at the concentrations exhibiting maximum effect on growth, exhibit comparable degrees of inhibition of shell growth in snails fed for rapid growth.

5. Diamox at concentrations up to 1.5 mg. per liter does not decrease the growth rate of snails induced to grow slowly by the feeding method employed though this concentration inhibited growth to a significant degree in rapidly growing snails.

6. The results are consistent with the hypothesis that carbonic anhydrase is essential in rapid shell development of some molluscs but may be insignificant in slow growth induced by poor feeding conditions, and afford additional evidence that the enzyme is significant in mollusc shell formation.

LITERATURE CITED

Abolins-Krogis, A., 1958. The morphological and chemical characteristics of organic crystals in the regenerating shell of *Helix pomatia* L. *Acta Zool.*, **39**: 19–38.

Berliner, R. W., and J. Orloff, 1956. Carbonic anhydrase inhibitors. *Pharmacol. Rev.*, **8**: 137–174.

Clark, A. M., 1957. The distribution of carbonic anhydrase in the earthworm and snail. *Australian J. Sci.*, **19**: 205–206.

Florkin, M., and P. de Marchin, 1941. Distribution de l'anhydrase dans les tissus de l'anodante. *Arch. Internat. Physiol.*, **51**: 130–132.

Freeman, J. A., and K. M. Wilbur, 1948. Carbonic anhydrase in molluscs. *Biol. Bull.*, **94**: 55–59.

Ikinago, K., 1954. The metabolism of the pearl oyster, *Pinctada martensii*—I. On the carbonic anhydrase. *Bull. Japanese Soc. Fisheries*, **19**: 925–928. (In Japanese with English summary.

Kawai, K., 1954a. Carbonic anhydrase in pearl oyster. I. Distribution and some properties of the enzyme. *Mem. Coll. Sci., Univ. Kyoto., Ser. B*, **21**: 39–44.

Kawai, K., 1954b. Carbonic anhydrase in the fresh-water mussel (*Hyriopsis schlegelii*) and physiological significance of the enzyme. *Japanese J. Mal.* **18**: 39–46. (In Japanese with English summary.)

KREBS, H. A., 1948. Inhibition of carbonic anhydrase by sulphonamides. *Biochem. J.*, **42**: 525–528.

KRUSKAL, W. H., AND W. A. WALLIS, 1952. Use of ranks in one-criterion variance analysis. *J. Amer. Statist. Assc.*, **47**: 583–621.

LARRANETA, M. G., AND F. PONZ, 1954. Alkaline phosphatase and carbonic anhydrase in some gastropods. *Rev. Espan. Fisiol.*, **10**: 225–234.

MAETZ, J., 1946. L'Activité anhydrasique de quelques tissus d'Invertébrés. *Bull. Inst. Oceanogr.*, **43**: 1–20.

MANN, H. B., AND D. R. WHITNEY, 1947. On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Statist.*, **18**: 50–60.

MANN, T., AND D. KEILIN, 1940. Sulphanilamide as a specific inhibitor of carbonic anhydrase. *Nature*, **146**: 164–165.

MELDRUM, N. U., AND F. J. W. ROUGHTON, 1933. Carbonic anhydrase. Its preparation and properties. *J. Physiol.*, **80**: 113–170.

MILLER, W. H., A. A. DESSERT AND R. O. ROBLIN, JR., 1950. Heterocyclic sulfonamides as carbonic anhydrase inhibitors. *J. Amer. Chem. Soc.*, **72**: 4894–4896.

STOLKOWSKI, J., 1951. Essai sur le déterminisme des formes minérologiques du calcaire ches les êtres vivants (calcaires coquilliers). *Ann. Inst. Oceanogr.*, **26**: 1–113.

TSUJII, T., AND A. MACHIDA, 1953. Carbonic anhydrase and the formation of pearls. *Kagaku*, **23**: 148.

WILBUR, K. M., AND N. G. ANDERSON, 1950. Carbonic anhydrase and growth in oyster and Busycon. *Biol. Bull.*, **98**: 19–24.

WILBUR, K. M., AND L. H. JODREY, 1955. Studies on shell formation. V. The inhibition of shell formation by carbonic anhydrase inhibitors. *Biol. Bull.*, **108**: 359–365.

THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. III. CALCIFICATION RATE AS A FUNCTION OF COLONY WEIGHT AND TOTAL NITROGEN CONTENT IN THE REEF CORAL MANICINA AREOLATA (LINNAEUS)

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This paper deals with an experimental study of the relationship between colony size, total nitrogen content, surface area and rate of calcification in the reef-building coral *Manicina areolata* (Linnaeus).

Tamura and Hada (1932) stated that coral growth ceases after certain colony sizes are attained. The Stephensons (1933) reported that the percentage annual increase in surface area and diameter was much greater in small colonies than in large ones. No attempt was made by these authors to determine whether the faster growth rate of younger corals was due to some inherent physiological factor, or whether it was due to the increasing disproportion of skeletal mineral matter, *e.g.* volume, in relation to the surface area occupied by the polypal mass, as corals get larger and older. Abe (1937) found that freshly settled larvae of *Fungia actiniformis* grew about three times faster between the first and fifth than from the sixth to the sixteenth days. Motoda (1940) believed that the decrease in the growth rate of the reef-building coral *Goniastrea aspera* with increase in colony diameter was the result of physiological senescence, and the progressive restriction of surface area.

To investigate the relationship of colony size on total nitrogen content and on the calcification rate in a reef coral, we chose *Manicina areolata* as the experimental animal. This species is plentiful on nearby reefs. Colonies of all sizes were obtained in numbers and even the largest were small enough to fit conveniently into our experimental vessels. *Manicina areolata* is found on the coral sand, shingle and grass bottom environments which are associated with the reef tract south of the town of Port Royal, Jamaica. This species is unique among West Indian reef-building Scleractinia in that it spends only a relatively short initial part of its life cycle attached by a stalk to small bits of shingle, shells or other corals. On reaching weights of approximately ten grams, the colonies usually break free, probably by accident, and fall unattached to the bottom where they continue to grow and proliferate.

Procedure

Colonies of *Manicina areolata*, weighing between 0.047 and 148.5 grams wet, were collected on 11 May 1959 from the lagoon of Drunkenman's Cay, without

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exposing them to contact with air. These were taken to the laboratory and kept for two days in large, well illuminated tanks provided with fresh circulating sea water to acclimatise them to their new environment. Colonies showing obvious damage, infestations of *Pyrgoma,* worms or other boring animals were rejected. To avoid injury, those of the smaller colonies which were still growing on pieces of rock or shell were not removed from their attachments.

After acclimatisation, the colonies were transferred, still under water, into large polyethylene vessels which were calibrated to contain three and a half liters. To this volume of unfiltered sea water was added an amount of buffered $Ca^{45}CL_2$[2] sufficient to give a specific activity of about 10^4 counts per minute per milligram of calcium in the aquatic medium. Illumination was provided by a twin bank of 40-watt daylight fluorescent tubes located about 12 inches above the water level. The water was stirred and aerated by a fine stream of wet air forced through a sintered glass filter stick which served as airstone. The pH of the medium increased from 8.20 to 8.34 during the experimental runs, and the water temperature fluctuated between 29.3° C. and 27.5° C. The experiments were allowed to run for 50 hours, water samples being taken after one, 24 and 48 hours for the measurement of the specific activity.

The runs were terminated by transferring the corals from the Ca^{45}-enriched water into fresh "cold" running sea water for two hours to allow washing out of all activity not deposited into the skeleton. The specimens were killed by putting them into a solution of slightly alkaline 4% formaldehyde. Later, the corals were placed on filter paper to remove the adherent fixative. Algae and other encrusting organisms were carefully scraped away from the non-living base of each colony, and the length, width and weight were recorded for individual specimens. The colonies were then dissolved in separate flasks containing concentrated HCl, the volume of which was adjusted to the weight of the coral. After solution of the mineral matter the tissue was homogenised with a Potter grinder, and the final volume of the suspension carefully determined. The total nitrogen contained in each colony was calculated from replicate 3-ml. aliquots treated according to the Kjeldahl method.

The weight of calcium laid down during the experiment by each specimen was determined from the amount of calcium^{45} incorporated and the mean specific activity of the water in which the corals were run, according to the method described in a previous paper of this series (Goreau, 1959a). The final results were expressed in terms of weight of calcium deposited per gram of wet weight, *e.g.* mg. Ca $gm.^{-1}$, or as the amount of calcium deposited per milligram of nitrogen, *e.g.* mg. Ca mg. N^{-1}. The latter will be called the "specific calcification rate," and the use of the term "growth" in this paper is taken to mean increase in weight due to calcium carbonate deposition.

Besides using normal colonies of *M. areolata* containing large numbers of zooxanthellae, experiments were run concurrently, and under identical conditions, on a series of five colonies which had previously been grown in darkness for three months to cause complete loss of the zooxanthellae and boring algae. The coenosarc of these specimens was colourless and quite translucent in the expanded condition.

[2] Supplied as Ca-45-P-2 by the Oak Ridge National Laboratory.

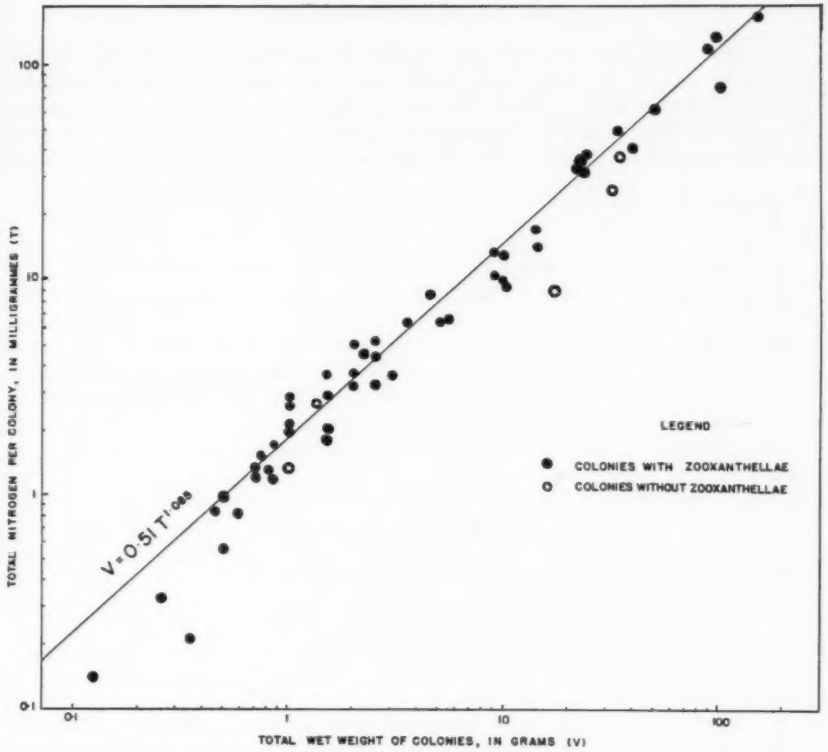

FIGURE 1. The relation between wet weight and total nitrogen content in colonies of the reef coral *Manicina areolata*. The solid circles represent colonies with zooxanthellae, the open circles are from colonies lacking zooxanthellae. The regression line was calculated by the method of least squares using the data from normal colonies only. The slope of the line is very close to unity, thus indicating that the volume, as measured by the weight, and the surface area, as measured by the total nitrogen, increase at the same rate. The significance of this is explained in the text. The position of the open circles near the regression line indicates that in comparison with normal colonies of the same weight, the nitrogen content of the bleached colonies is not significantly reduced by the absence of both zooxanthellae and boring algae.

In one colony a faint green discolouration was seen in the skeleton, indicating that some boring algae were still present although zooxanthellae were absent.

Dead colonies of *M. areolata* of different sizes, from which all tissues had been removed by maceration, were run in each series to test the amount of equilibrium exchange of calcium between the bare skeleton and the aquatic medium. No corrections were necessary for exchange since this occurred at an extremely low level in comparison with active deposition of calcium by living colonies. There was also no correlation between equilibrium exchange rates and the weight of the colonies used.

Results and Observations

We found that the total nitrogen content of specimens of *Manicina areolata* was directly related to their wet weight over the range of sizes investigated. This is shown in Figure 1 where the wet weight of the corals is plotted against their total nitrogen content. Logarithmic scales are used because of the large numerical range of the values. The nitrogen content of the smallest colonies tested

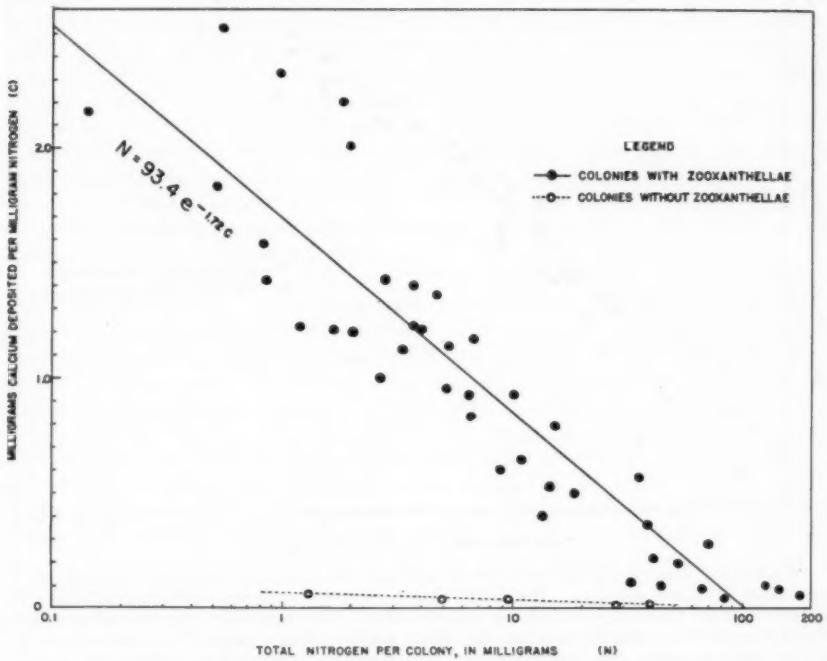

FIGURE 2. Graph showing the relation between the specific calcification rate and the total nitrogen in colonies of *Manicina areolata*. Corals with zooxanthellae are represented by solid circles, the bleached corals by open circles. The slope of the solid regression line was calculated by the method of least squares whereas the lower broken line was fitted by eye. These curves show that calcium is deposited at a progressively slower rate as the size of the colonies increases. The specific calcification rate of corals with zooxanthellae was about 30 times higher than that of bleached colonies of similar size.

was too low to determine by our Kjeldahl method; hence data from these were omitted from Figures 1 and 2.

The position of the points in Figure 1 indicates a good linear correlation between the wet weight V and the total nitrogen T. The regression line was fitted by the method of least squares and yields the equation,

$$V = 0.51\ T^{1.085} \qquad (1)$$

for the relation between V and T. The power of T is very nearly unity, which indicates that the ratio of wet weight to the total nitrogen is more or less constant over a large range of colony sizes. This means that, in individuals of *M. areolata* weighing between 0.1 and 148.5 grams, the amount of living tissue remains approximately the same in proportion to the total mass of skeletal calcium carbonate.

In colonies lacking the zooxanthellae, the relation between the weight of the individual specimens and their total nitrogen content does not appear to differ significantly from that of normal colonies of similar weight, as shown by the data plotted in Figure 1. Our results for *M. areolata* conflict with the assertion by Odum and Odum (1955) that the average living reef coral colony contains three times as much plant matter as animal matter. Bleached *Manicina areolata*, which lacked both zooxanthellae and boring algae, did not show the expected decrease in the protein nitrogen to weight ratio as compared with normal colonies of the same size. If these corals were to contain as much plant matter as stated by the Odums, then the nitrogen content of the bleached and zooxanthella-less corals should have been much less, size for size, than that of normal colonies. The fact that it was nearly the same would imply that the aggregate plant biomass associated with *M. areolata* is very much less than that found by the Odums for other species of corals.

With increasing size, we observed a striking fall in the calcification rate. The smallest colony tested, weighing 0.047 gram wet, deposited 92 mg. Ca gm. coral^{-1} hr.$^{-1}$ whereas the largest, weighing 148.5 grams wet, deposited only 1.28 mg. Ca gm. coral^{-1} hr.$^{-1}$. This is a 76-fold difference. The above calcification rates are given in terms of weight rather than nitrogen because the smallest colonies used contained too little nitrogen to be reliably measured by our Kjeldahl method.

On the whole, the relationship between the specific calcification rate C to the total colony nitrogen content N, as plotted in Figure 2, is given by the equation,

$$N = 93.4\ e^{-1.72\ C} \tag{2}$$

whereas the relation between calcification rate per gram of coral W to the total wet weight V, not shown in the figure, is given by the equation,

$$V = 63.8\ e^{-1.55\ W}. \tag{3}$$

These formulae show that the growth rate, as expressed in terms of the rate of calcium deposition, decays as an exponential function with increase in size. Similar but less marked trends were observed in colonies lacking zooxanthellae, but in these the calcification rate, size for size, was 30 times less than that of normal colonies of *M. areolata*.

The intercept of the regression line with the x-axis in Figure 2 implies that colonies having more than about 102 mg. total nitrogen do not grow any more. There is no evidence for this, and the decremental relationship cited above may hold only for colonies below a certain size range. Our results show that much larger colonies still grow slowly, but we do not yet have enough data to determine what kind of relationship, if any, may be applied to describe growth versus size in such cases.

For corresponding colony sizes, the calcification rates observed in these experiments on *M. areolata* with zooxanthellae were similar to those previously re-

ported for this species under field conditions (Goreau and Goreau, 1959), but growth rates of the zooxanthella-less specimens were lower by a factor of about 1.5. A possible reason for the discrepancy is that the bleached *M. areolata* used in the present experimental series had been grown in laboratory tanks in darkness for three months whereas those used in our previous study were found growing in the decolourised state under a coral head in the natural environment of the reef. Under these conditions, the growth rate of the naturally bleached colonies may have been faster because of their better nutritional status.

Discussion

In the coral *Manicina areolata* there is an approximate one to one relationship between the wet weight of whole colonies and their protein nitrogen content, and the specific calcification rate is inversely related to the weight, *i.e.,* size, of the individual corals.

The linear relationship between colony mass and the amount of protein nitrogen in specimens, with and without zooxanthellae, is extremely interesting. Except for the boring algae, all living tissue in scleractinian corals occurs outside the surface of the skeleton. Therefore, the amount of nitrogen per colony will be a function of the total surface area covered by the living polyps. In massive hemispheroidal corals, an increase in the colony size causes the weight, *i.e.,* mass of skeletal calcium carbonate, to increase in proportion to the cube of the radius, whereas the tissue mass, *i.e.,* the surface area, increases only in proportion to the square of the radius. The relationship, which has been demonstrated by Kawaguti (1941) for the massive coral *Goniastrea aspera,* does not hold for *M. aerolata* where the volume and surface area increase in direct proportion to each other. Inspection of *M. areolata* colonies of various sizes shows why this is so. Very small young individuals are shaped like simple cups. These grow in size and proliferate by incomplete intratentacular budding. This results in a lengthening of the tentacular ring and oral disk, along with an increase in the number of the stomodea. At first, this growth produces a simple linear elongation of the colony until the length is about twice the width. As growth continues with the budding of new stomodea, the valley becomes sinuous and the edge of the theca folds so that the whole colony presents a convoluted appearance which becomes increasingly complex as the coral grows larger. A typical growth series is shown in Figure 3. In this manner, the area, as measured by the protein nitrogen, and the volume, as measured by the weight of calcium carbonate, retain a one to one relationship to each other, as demonstrated by the slope of the regression line in Figure 1.

Such a large increase in the surface area relative to the weight is almost certainly adaptive response to the ecological conditions in which *M. areolata* lives. This coral is very common in shallow water; the larger colonies are usually found lying loose on sand or shingle bottom, frequently in association with the marine grass *Thalassia* (Goreau, 1959b). Our reef surveys have now shown that it is also found in large numbers on the sand and rubble bottom of canyons in the reef buttress zone at depths down to at least sixty feet where it is one of the few corals which successfully and consistently colonise this particular habitat (Goreau, unpublished field observations). It is therefore clear from its ecology that *M.*

areolata is adapted for life on unstable substrates where it is constantly exposed to the danger of burial by shifting sediments, or to being overturned by wave turbulence and currents. The species has a very efficient mechanism for ridding itself of sediment by means of flagellar currents and moving sheets of mucus. It is also one of the few corals possessing a well developed righting reaction. We have observed colonies weighing up to about 65 grams successfully right themselves after having been turned upside down. This was accomplished by a great swelling

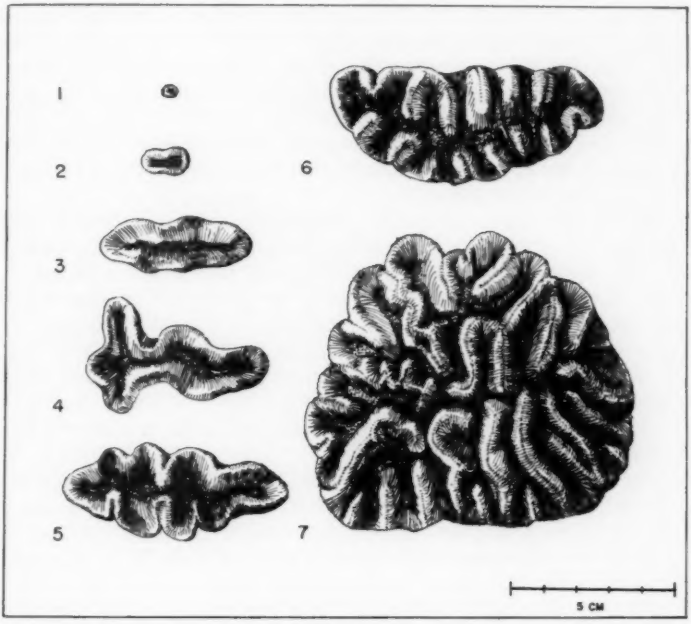

FIGURE 3. Relation between size and development of surface convolutions in a growth series of the coral *Manicina areolata*. The dry weights of these specimens are 0.09 gm., 0.74 gm., 6.2 gm., 10.3 gm., 21.4 gm., 58.7 gm., and 117 gm., respectively. The smallest individual shown here has a simple cup shape and only one stomodeum. Proliferation of additional stomodea by intratentacular budding at first produces linear elongation of the theca; later this becomes gradually more complex by formation of folds and branchings. Eventually, the typical convoluted brain-like surface pattern seen in the largest specimen is achieved. The continuous valleys of the specimens shown indicate that they are all clones descended from single planulae. Colonies of *M. areolata* which are formed through fusion of more than one planula invariably have a corresponding number of separate and distinct valley systems.

of the coenosarc through imbibition of water which was then vigorously ejected from the stomodea in the form of jets directed against the sand, thus undermining the colony on one side and rocking it until it was gradually righted in the normal position (Goreau, unpublished observations). It is almost certain that the ability of this coral to cleanse itself of sediment and to right itself if overturned must depend on its having a large surface area with respect to mass.

An interesting parallel is offered by the fungiid corals of the Indo-Pacific, which occupy an ecological niche in reefs very similar to that inhabited by *M. areolata* in the Western Atlantic, and which show striking analogies in their adaptive specialisations even though these corals belong to a different suborder of the Scleractinia. The young stages of both are attached to the substrate by stalks which subsequently break so that the older colonies are usually found free. In the fungiids, the stalk continuously buds off new colonies, in *Manicina* it does not. Both corals are commonly expanded in the day time; both increase their surface areas in more or less direct proportion to their mass, the fungiids doing this by assuming a flattened disk-like shape, *Manicina* by becoming more and more convoluted as its size increases. Both corals can inhabit environments where sedimentation and substrate instability prevent colonisation by most other species, largely through their innate ability to remain "afloat" on top of sand and shingle. In both, the highly sensitive youthful stages remain attached until they are heavy enough to resist displacement and turning over by anything but the strongest waves and currents.

Unlike the surface area, the specific calcification rate of *M. areolata* decreases with an increase in colony size. Motoda (1940, quoted by Wells, 1957) studied growth in *Goniastrea aspera* and concluded that progressive restriction of the surface area causes crowding of polyps in the center of the colony, thus slowing the growth rate as the spherical form is approached. We do not believe that these factors operate in the case of *M. areolata* since the surface area of this coral increases in direct proportion to the size. All colonies used in our experiments were descendants of single planulae as shown by their continuous valley patterns. There was no evidence that the stomodea in the center of the colonies were more crowded than those of the periphery.

Of all other variables which could influence the calcification rate with increasing age, the most obvious are the zooxanthellae. These dinophycean algae occur in the gastroderm of all hermatypic corals as intracellular commensals, and are known to affect the metabolism and growth rates of their hosts in a number of important ways. The presence of photosynthesising zooxanthellae constitutes a most effective stimulus to the mechanism of calcification, causing a very large increase in its rate in all species of reef corals we have tested so far (Goreau and Goreau, 1959). This effect is probably mediated by a decrease in the HCO_3^- concentration at the site of calcification when the zooxanthellae are photosynthesising, *i.e.*, removing CO_2 from the system (Goreau, 1959a). As is shown by the data given above, the calcification rate of *M. areolata* without zooxanthellae falls to extremely low levels in comparison to colonies of the same size which contain the zooxanthellae; nevertheless the growth rate of the smallest of our bleached colonies was nearly the same as that of the largest of the normal colonies that were tested. There is no evidence that the low calcification rate we found in the large specimens was due to a systematic reduction in the number of zooxanthellae with increasing age or size. On the contrary, large colonies of *M. areolata* are usually a darker brownish green than the young colonies which are mostly pale greenish brown in colour. Histological cross-sections of the oral disk show that there are about half as many zooxanthellae in the average thickness of the gastrodermis of small colonies about one centimeter long than in larger colonies about ten centimeters long. This means that the younger smaller colonies seem to have

fewer zooxanthellae per unit of weight than older larger colonies. The precise nature of this relationship is now being investigated in our laboratory. The decrease in calcification rate with increasing size is perhaps not due primarily to any variation in the number of zooxanthellae, but it occurs independently, as shown by our data on corals without zooxanthellae, in which this effect was also observed though it was much reduced in amplitude.

The mechanism by which age affects growth and other metabolic processes (*cf.* Brody, 1945) is not well understood. Although the possibility is suggested by our experimental results on *M. areolata*, it is still not clear whether other corals undergo a true process of senescence if all environmental factors remain optimal. There are probably very great individual variations in this respect among the different species of reef-building corals, as is indicated by the enormous variations in the size of some of them. Single colonies weighing several hundred tons are not uncommon. Many coral colonies are clones which are descended from single planulae. In these a progressive reduction in the speed of growth processes would be expected with increasing age of the coral. Similar phenomena have long been recognised, for example, in protozoa such as *Paramecium* and *Uroleptus* (Calkins, 1926) in which clonal ageing results in gradual failure of cell division.

There are almost certainly exceptions to this among the corals. For example, our observations on the branching West Indian staghorn coral, *Acropora cervicornis*, suggest the possibility that there is no reduction in the over-all growth rate with increasing size and/or age. In this species most of the growth takes place in the initial few centimeters of the branch tips occupied by large pale terminal polyps which have a far higher calcification rate than the much smaller lateral polyps immediately behind (Goreau and Goreau, 1959). These fast growing apical polyps are often seen to differentiate apparently in a spontaneous way, giving rise to new branches in parts of colonies which are already comparatively old and where the calcification rate is very low. The ability of these corals to grow new branches whenever and wherever needed must confer upon them an enormous adaptive advantage, since this enables them to respond rapidly to damage by waves and sediment. By using some of the lower branches as stilts to hold the main part of the colony off the bottom, it becomes possible for *A. palmata* to colonise sandy unstable habitats which would normally be inaccessible to most other corals having a more determinate pattern of growth.

This work was supported by grant G-4017 of the National Science Foundation to the New York Zoological Society, and by institutional funds from the University College of the West Indies. We wish to thank Professor D. M. Steven for putting at our disposal the facilities of the University's Marine Station at Port Royal for some of the experiments described above. We also record our grateful acknowledgment to Dr. R. C. Read and Mr. D. M. Hum of the University College Department of Mathematics for their advice on the statistical treatment of some of the data.

Summary and Conclusions

1. A study was made of the relation of colony weight to the total nitrogen content and calcification rate in the reef coral *Manicina areolata* (Linnaeus). Colonies weighing between 0.047 and 148.5 grams were used in our experiments.

2. In *Manicina areolata*, the ratio of the surface area, as measured by the total nitrogen, and the volume, as determined by the weight of the coral, remained nearly constant over the range of colony sizes tested. This is due to the fact that the surface of the coral becomes more and more folded as the colony grows older and larger. In those massive hemispheroidal corals which lack convoluted surfaces the increase in area would occur as a function of the square of the radius whereas the volume would increase as a function of the cube of the radius. It is suggested that the disproportionately large increase in the surface area of *M. areolata* with respect to growth in size, or volume, is an adaptive specialisation which aids this coral to colonise sandy or otherwise unstable bottom substrates, to clear itself more rapidly of sediment by means of flagellar currents, and to right itself if overturned by waves or currents.

3. The specific calcification rate of the smallest colonies was about 76 times greater than that observed in the biggest specimens of our series. The slopes of the plotted curves indicate that the growth rate decays as an exponential function of the size, *i.e.* the age of the coral, which can be described by a relationship of the type $X = Ae^{-\lambda Y}$ where X = total colony size, weight or nitrogen content, and Y = the amount of calcium deposited per unit weight or nitrogen. The growth rate of the largest colonies was somewhat greater than expected, and there is reason to believe that the relationship given above may hold only for colonies weighing less than about 100 grams.

4. Similar but much less marked decreases of the specific calcification rate with size were observed in *M. areolata* colonies which lacked zooxanthellae. It is suggested that, although the zooxanthellae are powerful stimulants to the calcification reaction, they are not as such responsible for the decay of the growth rate with increasing size and/or age.

5. The causes for the reduction in specific calcification rates with age are not understood, but may be due to an effect of senescence, similar in principle to the reduction in cell division which has been found to occur in old protozoan clones. Evidence is presented that some of the branching acroporid corals may not undergo a slowing of growth with age since these are able to sprout new fast growing branches even from the older parts of the colony. The survival value of this is discussed.

LITERATURE CITED

ABE, N., 1937. Post larval development of the coral *Fungia actiniformis* var. *polawensis* Doederlein. *Palao Trop. Biol. Sta. Stud.*, **1**: 73–93.

BRODY, S., 1945. Bioenergetics and Growth. New York, Reinhold Publishing Company, pp. 1023.

CALKINS, G. N., 1926. The Biology of the Protozoa. Philadelphia, Lea and Febiger, pp. 502.

GOREAU, T. F., 1959a. The physiology of skeleton formation in corals. I. A method for measuring the rate of calcium deposition under different conditions. *Biol. Bull.*, **116**: 59–75.

GOREAU, T. F., 1959b. The ecology of Jamaican coral reefs. I. Species composition and zonation. *Ecology*, **40**: 67–90.

GOREAU, T. F., AND N. I. GOREAU, 1959. The physiology of skeleton formation in corals. II. Calcium deposition by hermatypic corals under various conditions in the reef. *Biol. Bull.*, **117**: 239–247.

KAWAGUTI, S., 1941. On the physiology of reef corals. IV. The growth of *Goniastrea aspera* measured from numerical and areal increase of calyces. *Palao Trop. Biol. Sta. Stud.*, **2**: 309–317.

MOTODA, S., 1940. A study of growth rate in the massive reef coral *Goniastrea aspera* Verrill. *Palao Trop. Biol. Sta. Stud.*, **2**: 1–6.

ODUM, H. T., AND E. P. ODUM, 1955. Trophic structure and productivity of a windward coral reef community on Eniwetok Atoll. *Ecol. Monogr.*, **25**: 291–320.

STEPHENSON, T. A., AND A. STEPHENSON, 1933. Growth and asexual reproduction in corals. *Gt. Barrier Reef Exped. Sci. Rep.*, **3**: 167–217.

TAMURA, T., AND Y. HADA, 1932. The growth rate of reef building corals inhabiting the South Sea Island. *Tohoku Imp. Univ. Sci. Rep.*, **7** (4): 433–455.

WELLS, J. W., 1957. Annotated bibliography—Corals. *Geol. Soc. American Memoir*, **67** (1):1087–1104.

THE ACTION OF VARIOUS GOITROGENS IN INHIBITING LOCALIZATION OF RADIOIODINE IN THE THYROID AND THYMUS GLANDS OF LARVAL TREE TOADS

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Because of the differential affinity of the thyroid gland for iodine, properly adjusted doses of radioactive iodine (I^{131}) can produce enough radiation to destroy the thyroid completely without seriously damaging surrounding tissues. Immersion of tadpoles of *Hyla versicolor* at midlarval stages for 24 hours in a solution containing 20 μc./ml. of I^{131} results in rapid radiation damage to the thyroid so that no functional remnants are present after 10 days (Dent and Hunt, 1952). Administration of certain chemical substances known to inhibit the thyroid's ability to concentrate or bind iodine might be expected to prevent the thyroid from receiving enough radiation to cause permanent damage. Rugh (1953) found treatment with thiouracil effective in preventing or decreasing radiation damage in the thyroids of *Triturus pyrrhogaster* given a single injection of I^{131} (25 μc.).

Dent and Hunt (1952) observed that, in the tadpole, iodine is bound in other organs, notably the thymus, to a lesser degree than in the thyroid. It was demonstrated by Lynn and Dent (1957) that phenylthiourea inhibits iodine binding in both the thyroid and the thymus.

There are several different types of thyroid inhibitors with diverse modes of action. In the present study we have shown that some of them differ in degree and duration of effect by testing their capacities for preventing or diminishing radiation damage to the thyroid and thymus during administration of a large dose of I^{131}.

Materials and Methods

Tadpoles of *Hyla versicolor* collected near Oak Ridge, Tennessee, were maintained in the laboratory at 21° ± 1° C. until they reached late limb bud stages (equivalent to stages III to V of the Taylor and Kollros (1946) series). At this time they were divided into control and various experimental groups. Drugs to be tested were dissolved in the spring water in which the experimental animals were raised. The tadpoles were fed crumbled pellets of Purina Rat Chow, an excess of food being available for ~ 8 hours each day. The culture media were changed daily.

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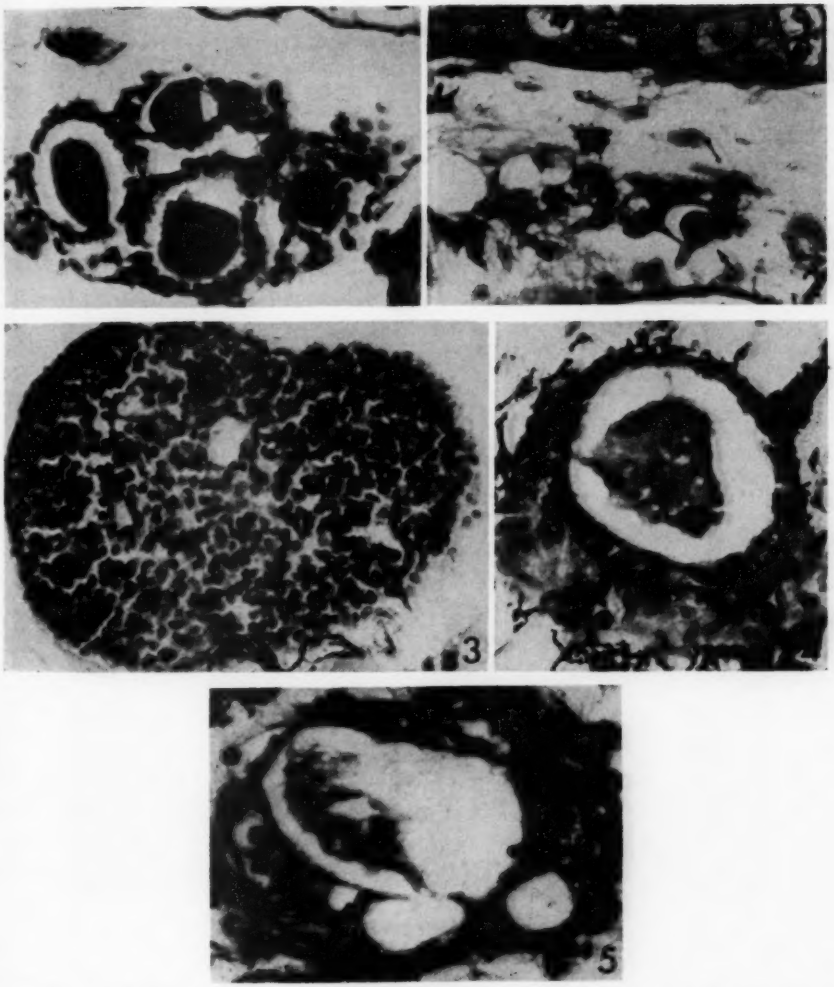
[3] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.



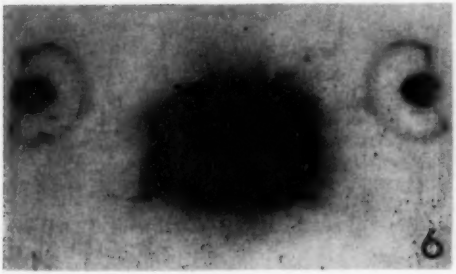
Figures 1 through 5 are photomicrographs of sections of larval specimens of *Hyla versicolor*.

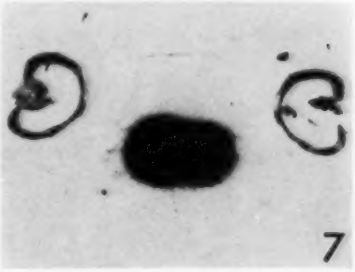
FIGURE 1. Thyroid gland from control specimen with histological evidence of moderate secretory activity.

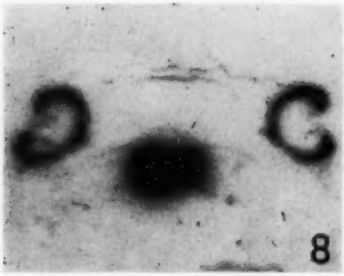
FIGURE 2. Arrow points to remnant of a thyroid follicle from an animal that received a thyroid-destroying dose of I^{131} and no goitrogen. Thyroid glands from most of the animals treated in this fashion were completely destroyed.

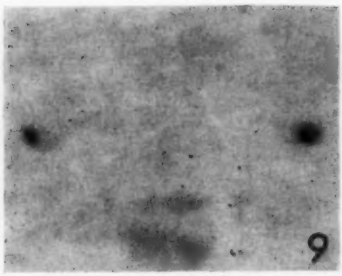
FIGURE 3. Thymus gland from control specimen. Note cords of cells and small sinus.

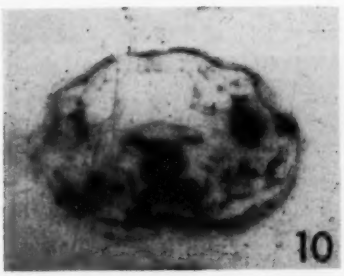
FIGURES 4 AND 5. Thymus glands showing severe radiation damage. The one in Figure 4 has a single cyst, the one in Figure 5 a large cyst and two smaller ones.

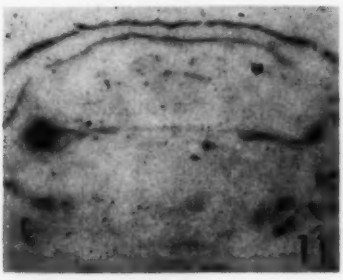












Figures 6 through 11 are photomicrographs of contact autoradiograms made from sections of larval specimens of *Hyla versicolor* treated with I^{131}.

FIGURE 6. Thyroid region of control tadpole after a tracer dose of I^{131}. The spots produced by the two lobes of the thyroid gland are fused into a single spot the size of which is many times that of the gland (compare size of images of eyes).

FIGURE 7. Thyroid protected from the histological damage of the thyroid-destroying dose of I^{131} by goitrogen and then given a tracer dose of I^{131}. The size of the spots indicates that the gland is functioning at a lesser rate than glands of control animals.

FIGURE 8. Thyroid protected from histological damage. No tracer dose was given. The iodine present is residual from the thyroid-destroying dose.

FIGURE 9. Thymus region of control animal given only a tracer dose of I^{131}. The lobes of the thymus have produced the spots on either side of the photograph. They are slightly larger than the sections of the gland that produced them. The double spot on the left represents concentrations of I^{131} in two sinuses. The diffuse spot in the lower center represents the heart.

After a 15-day period [4] of goitrogen administration one series of tadpoles was given a large (thyroid-destroying) dose of radioiodine by immersion for 24 hours in a solution containing 20 μc./ml. of I^{131}. They were then passed through two baths of spring water and maintained in spring water, with the usual daily feeding schedule, for 10 days. These animals and some not previously exposed to radioiodine were given a tracer dose of radioiodine by immersion for 24 hours in a solution of 1 μc./ml. of I^{131}. In a second series of animals, the same procedures were followed except that the tracer dose was omitted. All tadpoles were kept in spring water for an additional day and were then fixed in Bouin's fluid, embedded, and sectioned. Contact autoradiograms were prepared after the method of Dent and Hunt (1952) by apposing slides bearing sections of the larvae to Eastman medium-contrast lantern slide plates for an exposure time of 8 days. All sections were subsequently stained with Harris's hematoxylin and Ponceau de xylidine-orange II (Gray, 1952). Figures 1–5 are photomicrographs of sections of larval specimens. Figures 6–11 are photomicrographs of contact autoradiograms made from sections of larvae treated with I^{131}.

Results

1. *Thyroid gland*

(*a*) *Effects of radioiodine treatment on normal thyroid.* Tadpoles kept continuously in spring water and given only the tracer dose of I^{131} provided the basic controls in this experiment. Histological observations of thyroid glands from eight such animals indicated moderate secretory activity (Fig. 1). Large follicles were lined with cuboidal epithelium and contained much acidophilic colloid. Chromophobe droplets were present at the periphery of the colloid mass in most follicles. The marked ability of these thyroids to concentrate iodine is evidenced by an intense black spot on the autoradiographic plates over each lobe that fused with the spot representing its companion lobe (Fig. 6).

Administration of the large dose of I^{131} to nine tadpoles resulted in complete destruction of the thyroid in six of them. In the other three, tiny spots running through two or three sections on the autoradiograms corresponded to minute thyroid remnants on the slides. Two of these remnants consisted of isolated colloid masses with no surrounding cells, the other of several follicles with severely damaged epithelia (Fig. 2). These tadpoles, having received no drugs, constitute the experimental controls of the series receiving the thyroid-destroying dose of I^{131}. Table I summarizes the histological findings for all the experimental animals that received the large dose of I^{131}.

[4] In preliminary experiments, the period of treatment with phenylthiourea varied from 25 to 9 days. Within these limits the protective effect was not correlated with the period of goitrogen administration. The standard period used in subsequent experiments was 15 days.

Figure 10. Thymus region of animal given both thyroid-destroying and tracer doses of I^{131}. The large, dark spot on the right represents the right thymus; the left is not present in this section.

Figure 11. Thymus region of animal given only thyroid-destroying dose. Only a small part of the right thymus is present. The left thymus has produced a large spot, indicating considerable retention of I^{131}.

(*b*) *Effects of treatment with phenylthiourea.* In experiments performed to test the degree and duration of effect of phenylthiourea three variations in procedure were followed: (1) treatment with 0.01% phenylthiourea was continued during the 24-hour period of I^{131} administration, the I^{131} solution being made up in phenylthiourea solution; (2) treatment with phenylthiourea was stopped at the time of I^{131} administration, the I^{131} solution being made up in spring water; (3) treatment was stopped two days before I^{131} administration.

The thyroids of eight tadpoles given phenylthiourea before and during I^{131} treatment were indistinguishable histologically from those of the basic control tadpoles, although their physiological activity was reduced in that they produced autoradiograms about half the size of those produced by control thyroids (Fig. 7).

When phenylthiourea treatment was discontinued at the time I^{131} administration was begun, the protective value of the treatment was greatly decreased. Thyroids of five animals handled in this manner showed extensive radiation damage with destruction of cells, pyknosis of nuclei, and, in some regions, complete disruption

TABLE I

Degree of radiation damage in thyroid glands of tadpoles after immersion in solutions of goitrogens and subsequent immersion in solutions containing 20 μc./ml. of I^{131} (thyroids of controls immersed in iodine without goitrogen treatment were destroyed)

Time of removal from goitrogen solution	Drugs in which animals were immersed				
	Phenylthiourea (0.01%)	$KClO_4$ (0.01%)	KSCN (0.005%)	$KClO_3$ (0.005%)	KIO_3 (0.005%)
At end of I^{131} administration	0	0	0	0	0
At beginning of I^{131} administration	++	0	++	NT	NT
Two days before I^{131} administration	+++	0	+++	NT	NT

Legend: 0 = No histological evidence of radiation damage.
++ = Evidence of extensive radiation damage.
+++ = Evidence of complete or essentially complete destruction.
NT = Not tested.

of follicles. Three of these thyroids produced no autoradiographic images; small isolated spots corresponding to less seriously damaged follicles were present in the others.

Discontinuance of phenylthiourea treatment two days before I^{131} administration resulted in complete destruction of the thyroid.

(*c*) *Effects of treatment with perchlorate.* Three sets of experiments corresponding with those just described for phenylthiourea were carried out with 0.01% $KClO_4$.

In contrast with phenylthiourea, $KClO_4$ completely protected the thyroid not only when given during I^{131} administration, but even when discontinued two days earlier. No histological abnormalities were seen in thyroid sections of this series, and autoradiograms showed dense spots representing the thyroids. Like those from thyroids protected by phenylthiourea, the spots were somewhat smaller than in untreated controls but indicated, nevertheless, a relatively high I^{131} content.

Autoradiograms prepared from sections of animals that did not receive the tracer dose of radioiodine still showed small but definite spots for the thyroid (Fig. 8), indicating that a part of the heavy dose of radioiodine was retained in the protected glands.

Treatment with a lower concentration of $KClO_4$ (0.005%) gave similar results, indicating that these two concentrations are equally effective.

(*d*) *Effects of treatment with thiocyanate.* Although animals were treated with both 0.01% and 0.005% KSCN, the higher concentration was so toxic that only a few animals survived the experiment, and they were discarded. The lower concentration prevented morphological damage to the thyroid when treatment was continued during I^{131} administration. Cessation of treatment at the time I^{131} was given, however, resulted in extensive damage, only a few badly disrupted remnants of the thyroid being discernible. These remnants were sometimes represented by tiny spots on the autoradiograms. Cessation of pretreatment two days before I^{131} administration resulted in complete destruction of the thyroid.

(*e*) *Effects of treatment with chlorate and iodate.* A single series of experiments was run to test the effects of treatment with 0.005% $KClO_3$ and 0.005% KIO_3 that was continued during I^{131} administration. Under these conditions these substances prevented any visible histological damage to the thyroid. Autoradiograms showed large intense spots representing the glands.

2. *Thymus gland*

(*a*) *Effects of radioiodine on the thymus of control animals.* At the stage studied, the thymus gland is made up of loosely defined cords of cells surrounding small sinuses filled with lymphocytes and erythrocytes (Fig. 3) so that the gland presents a relatively compact appearance in section (Speidel, 1926; Dent and Hunt, 1952).

Administration of the large dose of I^{131} to previously untreated animals resulted in extensive damage to the thymus. In most of them the thymus was represented only by a single large vesicle with a few intact cells around the periphery (Fig. 4). Such glands produced much more intense spots on autoradiograms than did the normal thymus (Figs. 9 and 10). In the relatively few instances in which a number of separate vesicles were present (Fig. 5), rather than a single large one, the autoradiographic spots always corresponded to the vesicles. The material in these vesicles thus had a higher I^{131} content than the cells of the thymus.

(*b*) *Effects of treatment with goitrogens.* For all the preceding treatments discussed, histological and autoradiographic study was made of the thymus as well as the thyroid. Only one treatment proved completely effective in preventing thymus damage under the conditions of these experiments. Treatment with 0.01% $KClO_4$ continued during I^{131} administration prevented cystic change in the thymus in all nine animals treated. Judging from the extent of damage, similar treatment with 0.005% $KClO_4$ or 0.005% KSCN gave partial protection, but 0.005% $KClO_3$, 0.005% KIO_3 and 0.01% phenylthiourea gave none. No evidence of protection was seen in any tadpoles in which goitrogen treatment was discontinued before or at the time of I^{131} administration.

Any large vesicles present in the damaged thymus were represented by intense spots on autoradiograms. Because these spots were present whether or not a

tracer dose of I^{131} was given (Figs. 10 and 11), it is evident that they resulted primarily from residual iodine of the original dose.

Discussion

Work with mammals indicates that, with the exception of phenylthiourea, the goitrogens used in this study interfere with the thyroid's function by inhibiting its ability to concentrate iodide (Wyngaarden *et al.*, 1952). Phenylthiourea, however, permits iodide concentration but prevents its binding to protein (Pitt-Rivers, 1950; Roche and Michel, 1955). Under the experimental conditions of this investigation all these substances protected the thyroid against retaining sufficient I^{131} to cause histological damage to the gland, provided that the goitrogen treatment was continued during the administration of I^{131}, whereas only $KClO_4$ was effective when pretreatment was discontinued either two days before or immediately before I^{131} administration.

Longer periods between the administration of the large I^{131} dose and fixation of the specimens might produce progressive damage since even those thyroids that were considered to have been completely protected retained a large amount of I^{131} 10 days after the thyroid-destroying dose was given. In mice, damage resulting from a low dose (25 μc.) of I^{131} is not apparent until many months after the iodine has been injected (Dent, Gadsden and Furth, 1955). Our experiments, however, were directed toward testing the relative effectiveness and duration of effect of the various goitrogenic drugs and were not concerned with devising a method for protecting the thyroid from radiation damage. Continuation of the goitrogen treatment for some time after exposure to I^{131} would presumably provide complete protection.

It is also noteworthy that, in a number of the experiments in which the thyroid seemed completely undamaged histologically, autoradiograms nevertheless indicated that the gland's ability to take up I^{131} from the tracer dose was considerably decreased. This may be interpreted as evidence of physiological damage in the absence of histological changes, or it may indicate a residual inhibitory effect of the drug as seen in the rat after the administration of propyl thiouracil (Durbin *et al.*, 1957).

The only similar experiments with an amphibian are those of Rugh (1953), in which adult specimens of *Triturus pyrrhogaster* were given a series of four intraperitoneal injections of a 1% suspension of thiouracil over a period of 3 weeks. These animals and untreated controls then received an intraperitoneal injection of 25 μc. of I^{131}. The first indications of damage to the thyroid were seen in the controls after one month, and almost complete destruction had occurred by two months. The specimens given thiouracil showed no damage at one month, minor damage at two months, and clear signs of protection even at 7 months. Even more complete protection might have been afforded, Rugh pointed out, by continuing administration of the goitrogen after I^{131} treatment. Although our experiments were undertaken with a different aim and technique, our results are in accord with those of Rugh.

Our observations also confirm the earlier observation of Dent and Hunt (1952) that the amphibian thymus has a differential affinity for I^{131} and that cystic changes

occur in this gland when sufficient I^{131} is administered. The amount of I^{131} taken up by the thymus is apparently quite low compared with that concentrated by the thyroid. I^{131} concentration in cysts of damaged thymus tissue is apparently much higher than in normal cells of the thymus.

The protective action of goitrogenic drugs in the thymus differed from that in the thyroid. Only one treatment (0.01% $KClO_4$ continued during I^{131} administration) completely prevented cystic changes in the thymus. None of the drugs provided any protection for the thymus if their use was discontinued before I^{131} administration. These goitrogens are all believed to interfere with the thyroid's ability to take up iodine from the circulating blood. Since $KClO_4$ was the most effective inhibitor of I^{131} uptake by both thyroid and thymus, the same mechanism may be operative in both cases. The lesser degree of effectiveness of the drugs for the thymus compared with the thyroid may indicate that the thymus is damaged by lower radiation levels than is the thyroid.

Although protective to the thyroid, phenylthiourea proved to be completely without protective value in the thymus. Although phenylthiourea caused a decrease in I^{131} uptake by the thymus (Lynn and Dent, 1957), the present results indicated that this reduction is not sufficient to protect the thymus from damage by a thyroid-destroying dose of I^{131}.

Summary

1. Larvae of *Hyla versicolor* were immersed for 15 days in spring water containing phenylthiourea, $KClO_4$, KSCN, $KClO_3$, or KIO_3 followed by a 24-hour immersion in 20 μc./ml. of I^{131}, a thyroid-destroying dose.

2. When treatment with any one of these drugs was continued during the period of I^{131} administration, radiation damage to the thyroid was prevented.

3. The inhibitory effect of $KClO_4$ on the thyroid persisted even when treatment was discontinued two days before immersion in the I^{131} solution.

4. The effects of phenylthiourea and KSCN were dissipated more quickly since the thyroid showed extensive damage when treatment was stopped immediately before I^{131} administration and complete destruction when it was stopped two days before.

5. In control animals, the thyroid-destroying dose of I^{131} extensively damaged the thymus gland as well.

6. Of the drugs tested, only $KClO_4$ sufficiently inhibited the I^{131} uptake by the thymus to prevent damage, and only when it was present during the period of exposure to I^{131}.

Literature Cited

Dent, J. N., and E. L. Hunt, 1952. An autoradiographic study of iodine distribution in larvae and metamorphosing specimens of anura. *J. Exp. Zool.*, **121**: 79–98.

Dent, J. N., E. L. Gadsden and J. Furth, 1955. On the relation between thyroid depression and pituitary tumor induction in mice. *Cancer Res.*, **15**: 70–75.

Durbin, P. W., C. W. Asling, M. E. Johnston and J. G. Hamilton, 1957. The effect of propyl thiouracil on the recovery of rat thyroid gland from irradiation with astatine^{211}. *Anat. Rec.*, **129**: 17–37.

Gray, P., 1952. Handbook of Basic Microtechnique. Blakiston Company, Philadelphia.

Lynn, W. G., and J. N. Dent, 1957. Phenylthiourea treatment and binding of radioactive iodine in the tadpole. *Biol. Bull.*, **113**: 160–169.

PITT-RIVERS, R., 1950. Mode of action of antithyroid compounds. *Physiol. Rev.*, **30**: 194–205.

ROCHE, J., AND R. MICHEL, 1955. Nature, biosynthesis and metabolism of thyroid hormones. *Physiol. Rev.*, **35**: 583–610.

RUGH, R., 1953. Thiouracil modification of effect of radioiodine (I^{131}) on the thyroid. *Radiology*, **61**: 391–400.

SPEIDEL, C. C., 1926. Studies of hyperthyroidism. II. The significance of changes in the thymus glands of thyroid-treated frog tadpoles. *Amer. J. Anat.*, **37**: 141–157.

TAYLOR, A. C., AND J. J. KOLLROS, 1946. Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.*, **94**: 7–23.

WYNGAARDEN, J. B., B. M. WRIGHT AND P. WAYS, 1952. The effect of certain anions upon the accumulation and retention of iodide by the thyroid gland. *Endocrinology*, **50**: 537–539.

INVESTIGATION OF THE FERTILIZATION INHIBITING ACTION OF ARBACIA DERMAL SECRETION [1]

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Several investigations (Oshima, 1921; Pequegnat, 1948; Harvey, 1956; Metz, 1959a) have shown that *Arbacia* releases a fertilization inhibiting agent or agents when subjected to conditions of stress. The agent is present in a yellowish fluid called "dermal secretion" (Oshima, 1921) and is probably released from certain pigment-containing cells of the integument (Pequegnat, 1948). Indeed this dermal secretion is probably responsible for the fertilization inhibiting action ascribed to *Arbacia* blood by Lillie (1914).

Unfortunately, little information is as yet available concerning the mechanism by which the dermal secretion inhibits fertilization. In a previous study (Metz, 1959a) it was found that the dermal secretion inhibits the agglutination of *Arbacia* sperm by fertilizin, the specific sperm isoagglutinin obtained from the jelly layer surrounding eggs. This agglutination inhibiting action was found to result from an inactivation of the specific combining sites of the fertilizin that react with the antifertilizin of the sperm surface.

The parallel inhibiting action of dermal secretion on fertilizin agglutination of sperm and on fertilization suggests that the latter action may also result from inactivation of fertilizin combining sites. The present study was undertaken to investigate the mechanism of fertilization inhibition by the dermal secretion and particularly to determine if this action resulted from an inactivation of fertilizin. The experiments have been reported briefly elsewhere (Metz, 1959b). They indicate that fertilizin is not the site of inhibitor action.

Materials and Methods

Two species of sea urchins were used in the experiments, namely *Lytechinus variegatus* and *Arbacia punctulata*. Animals of the former species were obtained from the large population in the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida. The latter (*Arbacia*) were also obtained from this area, and from Panama City, Florida. This species was also obtained from the Woods Hole, Massachusetts, area and used at the Marine Biological Laboratory.

Gametes were obtained from *Lytechinus* by inducing the animals to shed by treatment with 0.5 *M* KCl. Gametes were obtained from *Arbacia* by electrical stimulation.

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Solutions of dermal secretion were obtained by immersing *Arbacia* in tap or distilled water for 1–3 minutes, rinsing them in sea water and collecting the yellowish fluid that drained from the animals (Metz, 1959a). The pH of such dermal secretion is about 7.5. The pH was adjusted to that of sea water (8.0–8.2) before use.

Results

In the presence of *Arbacia* dermal secretion both *Arbacia* and *Lytechinus* eggs fail to fertilize, even though these eggs are bombarded by highly motile spermatozoa. Such eggs fail to elevate fertilization membranes or show any indication of nuclear activity. Clearly, then, the dermal secretion blocks fertilization at its initial stages. The first step in analyzing the mechanism of this action of the

Table I

Percentage of eggs cleaving after insemination with Arbacia *sperm washed from dermal secretion*

Dilution of treated sperm	Dermal secretion—treated sperm		S.W.—treated sperm	
	Washed in S.W.	Re-suspended in D.S. supernatant	Washed in S.W.	Re-suspended in S.W. supernatant
1	100% (200+)	60% (103)	100% (200+)	100% (200+)
1/5	100% (200+)	100% (200+)	100% (200+)	100% (200+)
1/25	90% (128)	100% (200+)	99% (200+)	100% (200+)
1/125	62% (98)	100% (97)	97% (119)	100% (200+)
1/625	20% (109)	61% (116)	64% (108)	93% (123)

Figures in parenthesis are the number of eggs counted. The four mixtures were prepared in the following proportions: 0.5 ml. approximately 20% sperm suspension; 1.5 ml. D.S. or S.W. These mixtures were centrifuged and the sperm suspended in 1.5 ml. S.W. or in the supernatant. This procedure was repeated and all four sperm suspensions were finally diluted with 25 ml. S.W. (sperm "dilution 1" = approximately 0.4% undiluted semen). The suspensions were diluted serially as indicated. Two drops of unfertilized eggs were added to 4 ml. of each sperm dilution. In a control experiment eggs failed to fertilize when inseminated in the D.S. supernatant from the centrifuged sperm suspension.

dermal secretion was to determine if the agent acted upon the sperm, the egg, or both gametes.

Action on the sperm. In the previous studies (Pequegnat, 1948; Metz, 1959a) it was found that the dermal secretion from *Arbacia* markedly enhanced both motility and respiration of *Arbacia* sperm and that these effects were long lasting. Evidently, then, any fertilization inhibiting action on the sperm must result from some subtile effect such as "premature discharge" of the acrosomal filament or blocking of an essential receptor site on the sperm surface.

To test for such action *Arbacia* sperm were treated with dermal secretion, subsequently washed free of the solution by centrifugation and finally tested for fertilizing capacity. As seen in Table I, *Arbacia* sperm washed from dermal secretion are as effective in fertilizing eggs as sperm washed from sea water. Furthermore, dilution of dermal secretion-treated sperm with sea water restores fertilizing capacity. Thus (Table I) the sperm in dermal secretion gave only 60% fertilization at "dilution 1" but yielded 100% fertilization at higher dilutions.

This inhibition at "dilution 1" is attributed to an action of dermal secretion on the eggs. At the higher dilutions the dermal secretion is evidently diluted beyond the minimum inhibitory concentration. Finally, the data suggest that washing, as opposed to re-suspension in the supernatant, reduces the fertilizing capacity of control sperm. This is in accord with Hayashi's (1945) observations on the beneficial effect of seminal plasma on sperm. Results similar to those in Table I were obtained in a second experiment using *Arbacia* sperm and eggs. Comparable results were also obtained in two experiments with *Lytechinus* sperm and eggs using *Arbacia* dermal secretion. However, the results were not as striking in these experiments because the more delicate *Lytechinus* sperm does not survive centrifugation and re-suspension as well as *Arbacia* sperm.

It is evident from these experiments that dermal secretion does not inhibit fertilization by irreversible action on the sperm. Accordingly, attention was directed toward possible effects on the egg.

Action of dermal secretion on the egg. According to Pequegnat (1948) washing in sea water at least partially restores fertilizability to dermal secretion-treated *Arbacia* eggs. Harvey (1956, page 57), on the other hand, states that "it has been my experience that sometimes the eggs are not fertilizable, even after repeated washings with sea water." Observations in the present study indicated that reversibility of inhibition by washing varies from preparation to preparation. Eggs washed from some dermal secretion preparations fertilize readily whereas those from others show varying degrees of reduced fertilizability. This is evidenced in two ways. As seen in Table III, experiment 1, eggs washed from a dermal secretion preparation may approach 100 per cent fertilizability. However, substantially higher sperm concentrations are required to fertilize such eggs than are required for the controls. A second expression of reduced fertilizability is delayed cleavage. In some experiments *Arbacia* eggs washed from dermal secretion and subsequently inseminated fail to cleave synchronously with the controls. In these, first cleavages are asynchronous and delayed one or two divisions as compared to the controls.

These results indicate that the probability of a "successful sperm-egg contact" (Rothschild, 1956) is lower for the treated eggs than for the control eggs under comparable conditions. The reduced probability of a successful sperm-egg contact in turn could result from a correspondingly reduced number of receptor sites on the egg surface. In the case of cleavage delay the treated eggs may not fertilize at the moment of insemination because time is required for a successful sperm-egg contact to be made. The assumption is then that the egg develops at the normal rate and cleaves on schedule following a successful sperm-egg contact. This view is supported at least to the extent that fertilized eggs show no cleavage delay when placed in the dermal secretion as early as one to two minutes following insemination. However, eggs placed in dermal secretion a few seconds after insemination frequently show arrested fertilization membrane elevation and sperm entry (especially clear in *Lytechinus* eggs). These observations suggest an inhibition of the cortical reaction. In any event these experiments show that washing does not readily restore dermal secretion-treated eggs to the control level of fertilizability. Accordingly, it appears likely that the dermal secretion inhibits fertilization by an effect upon fertilizability of the eggs, as opposed to sperm. This view is supported by studies with enzymes as recorded in a later section.

The effect of dermal secretion on the formation of blebs by inseminated oocytes. The observations described above suggest that the dermal secretion inhibits, blocks or otherwise prevents an early step in fertilization. In a further test of this assumption dermal secretion was examined for its effect on the formation of blebs or papillae on oocytes when these cells are inseminated. As is well known (*e.g.*, Rothschild and Swann, 1949) sea urchin oocytes are not fertilizable. However, these cells, when exposed to sperm, form numerous blebs on their surfaces. Formation of such blebs has been attributed to an interaction between the sperm and oocyte surface. To test for the effect of dermal secretion on oocyte bleb formation, suspensions of eggs containing oocytes were placed in dermal secretion solutions, sperm was then added and the mixtures examined for bleb formation and compared with appropriate controls. Many such observations using both *Arbacia* and *Lytechinus* eggs show that in the presence of dermal secretion bleb formation does not result to a significant degree, whereas numerous blebs formed in the control oocytes. Some inhibitory reaction evidently results which prevents interaction of sperm and oocyte or blocks the bleb-forming response mechanism of the oocyte. In any event these observations support the view that the dermal secretion blocks an early step in fertilization.

In a further attempt to localize the site of action of the dermal secretion, the egg surface was subjected to mild dissection and then tested for sensitivity to the inhibiting action of the dermal secretion.

The effect of dermal secretion on fertilizability of acid-treated eggs. The first agent selected for such dissection was acid sea water. As is well known (Tyler, 1948) acid sea water dissolves the jelly surrounding the egg and thereby removes the fertilizin from the egg except possibly for a layer bound to the egg surface. In two experiments *Lytechinus* eggs were exposed to pH 4–5 sea water and in four experiments *Arbacia* eggs were exposed to pH 5 sea water to remove the jelly layer. These eggs were subsequently washed in sea water, treated with dermal secretion and in some cases washed again and tested for fertilizability over a wide range of sperm concentrations. In all experiments the acid-treated eggs showed the same sensitivity to the fertilization inhibiting action of dermal secretion as did control eggs. Table II gives two experiments, one using *Arbacia* eggs and the other *Lytechinus* eggs. In both experiments it is clear that acid-treated and sea water-treated eggs are both sensitive to the fertilization inhibiting action of the dermal secretion. The well known reduction in fertilizing capacity upon removal of jellies from eggs (Tyler, 1948) is not pronounced at the sperm concentrations employed but indications of this effect are seen in the experiment with *Lytechinus* eggs.

Since the sensitivity of eggs to the fertilization inhibiting action of the dermal secretion is not affected by acid treatment, it follows that the site of action of the dermal secretion is not the egg jelly. It is also presumably not the egg fertilizin since fertilizin constitutes the jelly. However, it is possible that a layer of fertilizin remains bound at the egg surface after the acid treatment and that this is an essential layer blocked by the dermal secretion (*e.g.* Tyler, 1948).

The effect of dermal secretion on fertilizability of eggs treated with proteolytic enzymes. Proteolytic enzymes were used to achieve a further mild dissection of the egg surface. As is well known (*e.g.* Tyler and Metz, 1955; Tyler, Monroy and Metz, 1956), eggs treated with proteolytic enzymes lack jellies, they fail to

form normal fertilization membranes, they become polyspermic readily and hybridize more readily than control eggs when inseminated with sperm of foreign species. The following proteolytic enzyme preparations were used: "crystalline protease" (Nutritional Biochemical Corporation) in 0.1% sea water solution; "crystalline trypsin" (Nutritional Biochemical Corporation) in 0.01% sea water solution. Enzymatic action of the trypsin was stopped prior to addition of dermal secretion by washing the eggs in an excess of trypsin inhibitor (ovomucoid, Nutritional Biochemical Corporation, 0.1% solution in sea water; or soybean trypsin inhibitor, Mann Research Corporation, 0.1% in sea water). In all, ten experiments were performed. These included five with *Arbacia* eggs, one using protease and four employing the combination of trypsin and trypsin inhibitor. Experiments

TABLE II

Effect of Arbacia *dermal secretion on fertilizability of acid-treated eggs as indicated by per cent cleavage*

	Sperm dilution	Acid-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Arbacia eggs	1	23% (124)	91% (141)	15% (114)	90% (200+)
	1/5	5% (158)	97% (107)	2% (148)	90% (200+)
	1/25	1% (200+)	90% (152)	1% (200+)	90% (200+)
	1/125	0% (200+)	81% (128)	0% (200+)	97% (105)
Lytechinus eegs	1	14.5% (124)	88% (148)	19% (114)	96% (119)
	1/6	6% (114)	97% (147)	4.5% (150)	98% (134)
	1/36	0% (200+)	46% (140)	1% (200+)	35% (130)
	1/216	1% (200+)	14% (180)	0% (200+)	74% (182)

Arbacia eggs: Jellies were removed by acidification to pH 5.4, eggs were then washed twice in 50-ml. samples of S.W., 0.5 ml. eggs was added to 2.4 ml. D.S. or S.W. and finally three-drop samples of these eggs were added to 5-ml. samples of *Arbacia* sperm. Sperm dilution 1 = 0.1%.

Lytechinus eggs: Jellies were removed by acidification of pH 3.9. Eggs were washed twice in S.W., 0.75 ml. eggs was then added to 2 ml. D.S. or S.W. They were subsequently washed twice in S.W. Four drops eggs were added to 5 ml. of *Lytechinus* sperm. Sperm dilution 1 = 0.66%. D.S.-treated eggs at sperm dilution 1 showed high incidence of polyspermy. Many of the uncleaved eggs contained multipolar spindles.

using *Lytechinus* eggs included four applying protease treatment and one using the trypsin-trypsin inhibitor treatment. In all ten of these experiments enzyme-treated eggs were subsequently treated with dermal secretion (or sea water), washed in sea water and inseminated. With this procedure the enzyme-treated eggs fertilized equally readily whether they received the dermal secretion or sea water treatment. Proteolytic enzyme treatment alone, like acid treatment, lowers the fertilizing capacity of the eggs (*e.g.* Tyler and Metz, 1955). In Table III the results of two of the ten experiments, one using *Arbacia* and the other *Lytechinus* eggs, are presented. In both experiments the fertilizability of sea water-treated eggs was markedly reduced by treatment with dermal secretion. Thus in experiment 1 to even approach comparable fertilization (100% of the eggs), over 125 times the control sperm concentration was required to fertilize the dermal secretion-

treated eggs. In experiment 2 the difference in sperm concentration (to achieve 1% fertilization) was greater than 1000-fold. On the other hand no significant difference is seen between the fertilizability of dermal secretion and sea water-treated eggs that had been pretreated with enzyme. Indeed, comparison of the enzyme-treated and sea water-treated eggs shows that on an absolute basis enzyme

TABLE III

Effect of proteolytic enzymes on sensitivity of eggs to the fertilization inhibiting action of Arbacia *dermal secretion*

	Sperm dilution	Enzyme-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Experiment 1 trypsin-trypsin inhibitor-treated *Arbacia* eggs	1	99% (149)	97% (154)	89% (158)	100% (200+)
	1/5	98% (151)	68% (187)	91%‡ (162)	100% (200+)
	1/25	42% (295)	21% (186)	3% (156)	100% (200+)
	1/125	8% (199)	5% (187)	<1% (200+)	100% (200+)
Experiment 2 Protease-treated *Lytechinus* eggs	1	94% (107) (82%)*	93% (97) (51%)*	<1% (200+)	93% (88) (6%)*
	1/6	87% (118) (52%)*	83% (87) (47%)*	0% (200+)	98% (96) (2%)*
	1/36	81% (131) (13%)*	12% (149) (0%)*	0% (200+)	65% (113) (<1%)*
	1/216	37% (169) (<1%)*	25% (128) (<1%)*	0% (200+)	35% (108) (0%)*
	1/1296	14% (133)	1% (200+)	0% (200+)	<3% (200+)

% = Per cent cleavage.

Experiment 1. Two-ml. samples of *Arbacia* eggs were added to 4-ml. samples of S.W. and 0.05% trypsin (in S.W.). After 45 minutes the eggs were washed twice in S.W., 1.5 ml. of the eggs were transferred to 10 ml. 0.1% ovomucoid in S.W. Subsequently the eggs were washed again, samples were treated with D.S. and S.W. and washed. Four drops eggs were added to 5 ml. *Arbacia* sperm dilution series. A duplicate control experiment omitting the ovomucoid treatment gave similar results. Sperm dilution 1 = 0.1% semen. Degree of polyspermy was not recorded in this experiment. Figures in parenthesis are the total number of eggs counted.

‡ Cleavage was delayed two divisions as compared to controls.

Experiment 2. Five-tenths-ml. samples of unfertilized *Lytechinus* eggs were placed in 5 ml. 0.1% protease solution or S.W. After 89 minutes 1-ml. samples of protease- and S.W.-treated eggs were each added to 1-ml. samples of D.S. and S.W. Twenty minutes later the eggs were washed in S.W. and 4-drop samples were added to 5 ml. *Lytechinus* sperm at the dilutions indicated. Sperm dilution 1 = 16% of undiluted semen, approximately 2×10^8 sperm/ml. in this case.

* Percentage of eggs counted showing the abnormal cleavage characteristic of polyspermy.

pretreatment results in considerable improvement in fertilizability following exposure to dermal secretion. This improvement is evidently limited at the level of the fertilizability of enzyme-treated control eggs.

In the experiments just described the enzyme-treated eggs were washed free of dermal secretion and inseminated in sea water. If the enzyme-treated eggs are

inseminated directly in the dermal secretion they fail to show the marked improvement in fertilizability. In the discussions given below this reservation concerning the loss of dermal secretion sensitivity following trypsin treatment is assumed.

Two possible explanations for the improving action of proteolytic enzyme pretreatment are evident. The enzyme could destroy the activity of the dermal secretion and thereby protect the eggs from this inhibitor, or the enzyme could act directly upon the egg. Even after several washings sufficient enzyme might be carried over or remain adsorbed to the egg to prevent the inhibition by destroying the dermal secretion. This explanation might suffice for the experiments with protease-treated eggs. However, prior to exposure to dermal secretion the trypsin-treated eggs were exposed to an amount of trypsin inhibitor (ovomucoid, four experiments with *Arbacia* eggs; soybean inhibitor, one experiment with *Lytechinus* eggs) sufficient to inhibit all trypsin activity. Furthermore, in one experiment the proteolytic enzyme treatment was applied after exposure of the egg to dermal secretion. This post-treatment with enzyme was as effective as pretreatment in rendering dermal secretion-treated eggs fertilizable. Furthermore, in preliminary experiments the fertilization inhibiting action of dermal secretion was not appreciably affected by long exposure to trypsin. Clearly, then, trypsin does not render eggs insensitive to dermal secretion by inactivating this inhibitor. The alternative, second possibility is that the enzyme destroys or removes some dermal secretion sensitive site or substance of the egg which is essential in normal fertilization. Such action in turn evidently exposes some alternative pathway for fertilization. This alternative pathway is reversibly inhibited by dermal secretion because enzyme-treated eggs fail to fertilize when inseminated in dermal secretion.

Relation of dermal secretion action to the block to polyspermy. The response of enzyme-treated eggs following dermal secretion treatment paralleled the enzyme-treated sea water controls not only quantitatively but also qualitatively. As is well known, eggs treated with proteolytic enzymes undergo polyspermic development at sperm concentrations considerably lower than that required to obtain polyspermy in normal eggs. This susceptibility to polyspermy was not confined to enzyme-treated control eggs. Thus, in the enzyme-pretreated group of experiment 2, Table III, dermal secretion-treated eggs yielded at least as high an incidence of abnormal (polyspermic) cleavage as the enzyme-treated sea water controls. This was true at all sperm dilutions.

These observations suggest that the site of action of dermal secretion may be associated in some way with the block to polyspermy. Accordingly it seemed of interest to test eggs pretreated with another polyspermy inducing agent for sensitivity to the fertilization inhibiting action of dermal secretion. Nicotine has long been known to be a very effective polyspermy inducing agent (see Rothschild, 1953; Hagström and Allen, 1956, for literature) and was therefore selected as the polyspermy inducing agent. Nicotine diluted to 2×10^{-3} in sea water proved to be optimal for inducing polyspermy in *Arbacia* eggs. However, in two experiments such treatment did not lower the sensitivity of *Arbacia* eggs to the fertilization inhibiting action of dermal secretion. When tested quantitatively over a wide range of sperm concentrations, the nicotine-pretreated eggs showed the same degree of fertilization inhibition as control eggs after exposure to dermal secretion. Apparently, then, the sensitivity of eggs to the dermal secretion is not invariably associated with the block to polyspermy.

Relation of the fertilization inhibiting action of dermal secretion to fertilizin. As suggested above, the fertilization inhibiting action of dermal secretion and its reversal by proteolytic enzymes is most readily explained by assuming a combination of the inhibitor with an essential egg substance. Such combination blocks the essential substance and prevents it from entering into reactions involved in the initial stages of fertilization. The substance that immediately suggests itself is fertilizin, and especially so since dermal secretion prevents combination of fertilizin with sperm by inactivating the sperm receptor sites of the fertilizin molecule (Metz, 1959a). If the dermal secretion inhibits fertilization by an essentially irreversible combination of the inhibitor with fertilizin at or near the egg surface, then the fertilization inhibiting action of the dermal secretion should be destroyed by addition of an excess of fertilizin.

TABLE IV

Effect of fertilizin on fertilization inhibiting action of dermal secretion

D.S. dilution	D.S. diluted in fertilizin		D.S. diluted in S.W.		S.W. + fertilizin		S.W. alone	
	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination	Cleavage	Sperm agglutination
1/2	0% (200+)	−	0% (200+)	−	99% (136)	++++	100% (200+)	+
1/10	0% (200+)	+++ ++++	0% (200+)	±				
1/50	13% (159)	++++	6% (140)	+				
1/250	97% (119)	++++	86% (131)	+				

Aliquots of a dermal secretion preparation were diluted serially in sea water and in fertilizin as indicated, two drops of unfertilized *Arbacia* eggs were then added to 0.8-ml. samples of each D.S. dilution, and finally 5 ml. 0.0003% *Arbacia* sperm were added to each sample. % = per cent cleavage; values in parenthesis are the number of eggs counted. To test for fertilizin excess in the mixtures 2-drop samples of the supernatant from each mixture were withdrawn after insemination of the eggs. These were mixed with 2 drops 1–2% sperm and the agglutinating action recorded on a − to ++++ scale in the table. The sperm agglutination titer of the original fertilizin solution was >729.

To test for such inactivation of the fertilization inhibiting action of the dermal secretion by fertilizin, three experiments using *Arbacia* eggs were performed. In these experiments dermal secretion was diluted serially in fertilizin, constant amounts of eggs and sperm were added to the mixtures and the percentage of cleavage determined and compared with appropriate controls. The mixtures were also tested for presence of excess fertilizin by examining for sperm agglutinating action. One experiment, typical of the three, is given in Table IV. As seen in the controls the concentration of sperm used was sufficient to fertilize 99% of the eggs. However, it was necessary to dilute the dermal secretion to 1/250 to approach this value. Furthermore, the dermal secretion inhibited fertilization to the same dilutions (1/50 to 1/250) whether diluted in fertilizin or sea water. Indeed, at dilution 1/50, five times the dilution required to achieve fertilizin excess, only 13% of the eggs fertilized. This parallel loss of fertilization inhibiting action

when diluted in fertilizin and sea water, combined with the demonstration of a fertilizin excess at 1/10 dilution, clearly shows that the fertilization inhibiting action of dermal secretion is not impaired by fertilizin. Evidently, then, fertilizin does not neutralize the fertilization inhibiting action of dermal secretion. Therefore, it seems unlikely that dermal secretion inhibits fertilization by combination with fertilizin.

It should be noted that fertilizin treatment alone did not have a marked effect on fertilization in these experiments. This is in keeping with previous observations on *Arbacia* (Tyler and Metz, 1955). However, fertilizin treatment does have a marked effect in some other species (Tyler, 1941).

Discussion

The experiments described here show that the dermal secretion from *Arbacia* inhibits fertilization by action on the egg, not the sperm. The agent presumably blocks an essential reaction or reactions in the initial stages of fertilization. One such response of the egg that is evidently blocked by the dermal secretion is the formation of the fertilization membrane. In fact, treatment with dermal secretion a few seconds after fertilization arrests membrane formation. Such eggs have membrane elevated over only a part of the egg surface. It will be of interest to determine if other cortical phenomena of fertilization, such as cortical granule discharge (Motomura, 1941; Runnström, 1949; Endo, 1952) and the changes in optical properties observed in dark field illumination, are also arrested by the dermal secretion. These observations should give some indication of whether the agent inhibits the propagation of the cortical responses of the egg or interferes only with the process of membrane elevation (see Metz, 1957, for interrelations of cortical phenomena).

Unfortunately, the relationship of the blebs formed by inseminated oocytes to stages of fertilization of normal eggs is somewhat obscure. Therefore, no detailed interpretation of such inhibition in terms of fertilization is warranted. However, the blebs suggest that some of the initial stages of fertilization have taken place. Accordingly, inhibition of bleb formation by the dermal secretion indicates that this agent can inhibit initial steps in fertilization. It is not unlikely that the dermal secretion inhibits essential steps in the interaction of the sperm and the eggs, as well as arresting manifestations of the propagated responses of the egg.

The fertilization inhibiting action of the dermal secretion presumably results from interaction of the inhibiting agent with some essential substance or substances of the egg. Such interaction evidently results in destruction or blockage of the essential substance. This in turn reduces the number of receptor sites on the egg and the probability of a successful sperm-egg contact. This view is consistent with the observation that inhibitor-treated eggs can sometimes be fertilized if inseminated with high sperm concentrations.

Cases of cleavage delay may also be explained in formal fashion as a reduction of the probability of a successful sperm-egg collision. Thus eggs with a reduced number of receptor sites should have a lower "fertilization rate" (*e.g.* Rothschild, 1956; Hagström and Hagström, 1954) than control eggs because of the increased time required for the less probable event. Another possibility is that cleavage delay is a manifestation of a reversal of inhibition such that washed dermal se-

cretion-treated eggs undergo a slow reversal of inhibition. It should be noted, however, that other factors such as delay in the propagated response of the egg have not been eliminated as explanations for delayed cleavage of dermal secretion-treated eggs.

The fertilization inhibiting interaction between the dermal secretion and the essential egg substance could result in enzymatic destruction of the latter. It appears more likely, however, that the inhibitor acts by relatively undissociable combination with an egg substance. This view is supported by the observation that washing sometimes results in at least partial restoration of fertilizability to dermal secretion-treated eggs. It is also consistent with the observed reversal of inhibition by proteolytic enzymes.

The identity of the egg substance or substances with which the dermal secretion combines to inhibit fertilization remains obscure. This material is evidently not the sperm isoagglutinin, fertilizin. Several lines of evidence support this view. Fertilizin constitutes the jelly layer surrounding the egg. Eggs from which this jelly has been removed by acid sea water treatment are dermal secretion sensitive. Evidently, then, fertilization inhibition is not mediated by action of the dermal secretion on the jelly. However, this experiment does not rule out the possibility of action on an acid-resistant layer of fertilizin bound to the egg surface (*e.g.* Tyler, 1941). Further "dissection" of the egg surface was achieved by treating eggs with proteolytic enzymes. Following such treatment the eggs were relatively insensitive to the fertilization inhibiting action of dermal secretion. Evidently the enzyme digests or otherwise eliminates the primary egg substance with which the dermal secretion combines. However, there is some question if even the relatively drastic action of proteolytic enzymes removes all the fertilizin from the egg surface (Tyler and Metz, 1955) in a reasonable time. As a final test for a role of fertilizin in the inhibition of fertilization by dermal secretion, fertilizin and dermal secretion were mixed and the mixtures were assayed for fertilization inhibiting action. The fertilizin had no effect on the inhibiting action of dermal secretion. This was true even when the fertilizin was present in large excess as measured by sperm agglutinating action.

In view of these results it appears that the dermal secretion does not inhibit fertilization by an action on fertilizin. This is of particular importance because previous studies (Metz, 1959a) have shown that dermal secretion of *Arbacia* destroys the sperm combining sites of the fertilizin molecule.

Apart from consideration of the nature of the egg substance involved in the inhibition, the reversal of inhibition by either "pre- or post-" treatment of eggs with proteolytic enzymes has interesting implications for other aspects of fertilization. According to the interpretation given above dermal secretion inhibits fertilization by combination with a substance that is essential for fertilization of the normal egg. Pretreatment of the egg with proteolytic enzymes removes this essential egg substance and renders the egg relatively insensitive to the inhibitor. Evidently coincident with the removal of the essential egg substance by enzyme, an alternative pathway(s) to fertilization is exposed. Whether this pathway resides in the sperm-egg attachment mechanism, the activation initiating mechanism or the propagative and cortical response mechanism remains to be established. However, it should be noted that the "alternative pathway" exposed by proteolytic

enzyme treatment is associated with a loss of the block to polyspermy and a reduction in fertilization specificity (Bohus Jensen, 1953; Hultin, 1948a and 1948b; Tyler and Metz, 1955) and is itself reversibly inhibited by dermal secretion. This last conclusion follows from the fact that enzyme-treated eggs must be washed free of dermal secretion before they will fertilize. Whatever the site of action of the proteolytic enzymes the action does not result in drastic visible effects on the unfertilized egg, for sections of trypsin-treated and control eggs are indistinguishable when seen with electron optics (Parpart, personal communication).

This investigation suggests the use of fertilization inhibiting agents as markers or labels for particular sites or substances involved in fertilization. Extension of the investigation to include detailed examination of other fertilization inhibitors should prove interesting for these may act at other sites. Indeed, the studies of Branham and Metz (1959) indicate that the inhibition of fertilizin agglutination of sperm by extracts from *Fucus* and by dermal secretion results from two quite different mechanisms. It will not be surprising if these two agents are found to inhibit fertilization by action at different sites in the complex of reactions that result in fertilization.

Summary

1. Sperm washed from *Arbacia* dermal secretion fertilize eggs as readily as sperm washed from sea water.

2. Eggs washed from *Arbacia* dermal secretion do not fertilize as readily as controls. At best such eggs require high sperm concentrations to achieve fertilization. In some experiments the treated eggs show a marked cleavage delay. It is concluded that the dermal secretion inhibits fertilization by action on the egg, not on the sperm.

3. Addition of dermal secretion to eggs several minutes after insemination has no effect on the development of the eggs. Similar treatment a few seconds after insemination results in arrest of fertilization membrane elevation and sperm penetration. The dermal secretion also inhibits the formation of blebs in inseminated oocytes. It is concluded that dermal secretion inhibits by blocking an initial stage(s) in fertilization.

4. Jellyless (acid-treated) eggs fail to fertilize after treatment with dermal secretion. They have undiminished sensitivity to the inhibiting action of the dermal secretion.

5. Proteolytic enzyme-treated eggs are relatively insensitive to the inhibitor. They fertilize as readily as controls after treatment with dermal secretion, provided they are washed free of this inhibitor. They fail to fertilize if inseminated in dermal secretion.

6. The dermal secretion has undiminished fertilization inhibiting action in the presence of an excess of fertilizin.

7. The experiments suggest that the dermal secretion inhibits fertilization by combining with some egg substance that is essential for fertilization of the normal egg. The egg substance is apparently not the sperm isoagglutinin, fertilizin. Treatment of the egg with proteolytic enzymes eliminates the essential egg substance and simultaneously exposes an alternative pathway to fertilization. The latter is reversibly inhibited by dermal secretion.

LITERATURE CITED

BOHUS JENSEN A., 1953. The effect of trypsin on the cross fertilizability of sea urchin eggs. *Exp. Cell Res.*, **5**: 325–328.

BRANHAM, J. M., AND C. B. METZ, 1959. Inhibition of fertilization and agglutination in *Arbacia* by extracts from *Fucus*. *Biol. Bull.*, **117**: 392–393.

ENDO, Y., 1952. The role of the cortical granules in the formation of the fertilization membrane in eggs from Japanese sea-urchins. *Exp. Cell Res.*, **3**: 406–418.

HAGSTRÖM, B. E., AND R. D. ALLEN, 1956. The mechanism of nicotine-induced polyspermy. *Exp. Cell Res.*, **10**: 14–23.

HAGSTRÖM, B., AND B. HAGSTRÖM, 1954. A method of determining the fertilization rate in sea-urchins. *Exp. Cell Res.*, **6**: 479–484.

HARVEY, E. B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, New Jersey.

HAYASHI, T., 1945. Dilution medium and survival of the spermatozoa of *Arbacia punctulata*. I. Effect of the medium on fertilizing power. *Biol. Bull.*, **89**: 162–179.

HULTIN, T., 1948a. Species specificity in fertilization reaction. I. The role of the vitelline membrane of sea-urchin eggs in species specificity. *Arkiv Zool.*, **40A**: 1–9.

HULTIN, T., 1948b. Species specificity in fertilization reaction. II. Influence of certain factors on the cross-fertilization capacity of *Arbacia lixula* (L.). *Arkiv Zool.*, **40A**: 1–8.

LILLIE, F. R., 1914. Studies of fertilization. VI. The mechanism of fertilization in *Arbacia*. *J. Exp. Zool.*, **16**: 523–588.

METZ, C. B., 1957. Mechanisms in fertilization. *In:* Physiological Triggers, T. H. Bullock, editor. American Physiological Society, Washington, D. C.

METZ, C. B., 1959a. Inhibition of fertilizin agglutination of sperm by the dermal secretion from *Arbacia*. *Biol. Bull.*, **116**: 472–483.

METZ, C. B., 1959b. Studies on the fertilization inhibiting action of *Arbacia* dermal secretion. *Biol. Bull.*, **117**: 398.

MOTOMURA, I., 1941. Materials of the fertilization in the eggs of Echinoderms. *Sci. Rep. Tohoku Imp. Univ., Ser. 4*, **16**: 345–363.

OSHIMA, H., 1921. Inhibitory effect of dermal secretion of the sea urchin upon the fertilizability of the egg. *Science*, **54**: 578–580.

PEQUEGNAT, W., 1948. Inhibition of fertilization in *Arbacia* by blood extract. *Biol. Bull.*, **95**: 69–82.

ROTHSCHILD, LORD, 1953. The fertilization reaction in the sea urchin. The induction of polyspermy by nicotine. *J. Exp. Biol.*, **30**: 57–67.

ROTHSCHILD, LORD, 1956. Fertilization. John Wiley and Sons, N. Y.

ROTHSCHILD, LORD, AND M. M. SWANN, 1949. The fertilization reaction in the sea-urchin egg. A propagated response to sperm attachment. *J. Exp. Biol.*, **26**: 164–176.

RUNNSTRÖM, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymol.*, **9**: 241–327.

TYLER, A., 1941. The role of fertilizin in the fertilization of eggs of the sea urchin and other animals. *Biol. Bull.*, **81**: 190–204.

TYLER, A., 1948. Fertilization and immunity. *Physiol. Rev.*, **28**: 180–219.

TYLER, A., AND C. B. METZ, 1955. Effects of fertilizin-treatment of sperm and trypsin-treatment of eggs of homologous and cross-fertilization in sea urchins. *Pubbl. Staz. Zool. Napoli*, **27**: 128–145.

TYLER, A., A. MONROY AND C. B. METZ, 1956. Fertilization of fertilized sea urchin eggs. *Biol. Bull.*, **110**: 184–195.

PROTEIN CHANGES IN DEVELOPMENT [1, 2]

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Biologists have long been in agreement with the thesis that morphological differentiation is either preceded by, or accompanied simultaneously by, an underlying chemical differentiation. In the testing of this hypothesis, considerable attention has been focused on the qualitative and quantitative changes occurring in the proteins of developing embryos.

The usual biochemical techniques have been employed to describe and to quantitate these changes. One should mention the solubility studies of sea urchin embryo proteins by Mirsky (1936), the electrophoresis of sea urchin embryo proteins by Monroy (1950), and the electrophoresis of amphibian embryo proteins by Flickinger and Nace (1952). Recently the serological technique has been used quite extensively; the precipitin test by Cooper (1948), Ebert (1951), Harding *et al.* (1955); the Oudin technique by Spar (1953) and by Cooper (1948).

In addition to these *in vitro* studies a number of *in vivo* studies have been carried out. Ebert (1953, 1955) and his co-workers have studied the effects of specific antisera on living chick embryos, and Tyler and Brookbank (1956) have studied the cytotoxic effects of specific antisera on sea urchin eggs. We have carried out similar studies on dissociated sponge cells (Spiegel, 1955). The results of these investigations have been extensively summarized by Nace (1955) and by Tyler (1957).

Briefly, the results obtained through the use of the serological techniques on amphibians and sea urchins are as follows. In the sea urchin, it has been demonstrated by Perlmann and Gustafson (1948) and Perlmann (1953) that new antigens appear during development. These new antigens were first detected in the gastrula stage. Harding *et al.* (1954, 1955), working with lethal hybrid sea urchin embryos, were able to detect paternal antigens at the blastula stage.

In the frog, it seems fairly well established from the work of Cooper (1946, 1948, 1950) and of Spar (1953) that new antigens are found in the blastula and gastrula stages. Ten Cate and Van Doorenmaalen (1950) and Flickinger *et al.* (1955) have shown that there is good correlation between the time of appearance of lens antigen and of the lens itself. Flickinger and Nace (1952) have detected new antigens in the tail-bud stage.

Clayton (1953) has further demonstrated that between the blastula and gastrula stage, before neurulation, and between neurulation and the formation of the tail-bud, a synthesis of antigenic material occurs. Ectoderm and archenteron roof contain fractions specific to themselves as well as having common antigens.

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[2] Part of this work was carried out in the Department of Biology, Colby College, Waterville, Maine.

A word of caution seems warranted when considering these results. It should be mentioned that Tyler (1957) has pointed out that in investigations of this kind (in which antisera produced against saline extracts—after suitable absorptions—have been used to detect changes in antigens) there is the uncertainty as to whether or not a particular antigenic structure remains associated with a saline-soluble constituent and as to whether or not it is available for reaction in specific absorption procedures. The appearance of new antigens or the loss of old antigens may represent a change in solubility, rather than the synthesis or destruction of an antigen. The above evidence, therefore, which has been represented as a synthesis of new material may rather reflect changes in solubility of pre-existing substances. This point will be more fully considered in the discussion of the results presented in this paper.

The study to be reported here involved the use of the technique of zone electrophoresis to follow the changes occurring in the proteins of developing embryos and to compare these changes with adult organ proteins.

Materials and Methods

I. *Preparation of extracts*

A. *Developmental stages.* Embryos of the frog, *Rana pipiens,* from the unfertilized egg through stage 21 (Shumway, 1940) were used. Eggs were obtained by pituitary injection of large adult females and fertilized by the usual method of stripping directly into a suspension of macerated testes in 10% Holtfreter's solution, pH 7.8. The embryos were then washed three times with sterile 10% Holtfreter's solution, pH 7.8, and incubated at 19–20° C. until the desired stage was attained. Extracts were prepared from embryo populations in which at least 95% of the embryos were of the same stage and developing normally. Regardless of the stage at which the extract was made, 100–200 embryos of each population were always carried through stage 22 and if subsequent development of these controls showed more than 5% of the embryos to be abnormal, the extract was discarded. For the unfertilized egg extracts, 100–200 eggs were fertilized and incubated as described above. If more than 5% of the embryos were abnormal, the extract was discarded.

The jelly of individuals of the desired stage was then removed by the papain-thioglycolate method of Spiegel (1951). After washing five times with sterile 10% Holtfreter's solution, pH 7.8, 500–1000 jelly-free embryos or eggs were allowed to settle out, under gravity, in a 50-ml. cellulose nitrate centrifuge tube. Excess supernatant fluid was removed by aspiration, leaving only the small amount of solution that was trapped in the interstices between individuals. The loosely packed embryos were frozen-thawed, homogenized in a glass homogenizer, and centrifuged at 23,000 *g* for 10 minutes. The clear supernatant fluid minus the fat layer was removed by aspiration and stored at − 20° C. until used. All developmental stages were treated in identical fashion. The temperature throughout the extraction procedure was 4° C.

B. *Adult organs.* The following organs were dissected from adult frogs of both sexes: brain, gastrocnemius muscle, and small intestine. These organs were then extracted by the identical method used for developmental stages. Adult blood

was obtained by cardiac puncture from adult male and female frogs, diluted with an equal amount of 10% Holtfreter's solution, pH 7.8, centrifuged at 23,000 *g* for 10 minutes, the supernatant fluid, strongly colored by hemoglobin, removed by aspiration, and stored at − 20° C. until used.

II. *Electrophoresis*

Before electrophoresis, the extracts were thawed and dialyzed in the cold vs. three changes of the electrophoretic buffer, Veronal, pH 8.6, $\mu = 0.05$, for 24 hours. The dialysates were centrifuged at 23,000 *g* for 10 minutes and 0.02 ml. of supernatant fluid was applied to filter paper strips (Spinco Part No. 300–028). The 8-strip Spinco Durrum-type electrophoresis cell and the Spinco Model R regulated power supply were used. Electrophoresis was carried out at room temperature using 25 ma constant current for 6 hours. Under these conditions rather remarkable reproducibility was obtained. Each stage or adult organ extract was prepared a minimum of five times and each preparation subjected to at least three separate runs, making a total of at least 15 electrophoretic runs per type of extract.

III. *Staining and scanning of paper strips*

At the completion of the run, the papers were dried at 115° C. for 30 minutes and stained by the rapid brom phenol blue procedure for proteins described by Durrum (1958). The strips were rinsed in methanol for 6 minutes, stained for 30 minutes in 0.1% brom phenol blue in methanol, followed by three rinses in 5% acetic acid of 6 minutes duration per rinse. They were then blotted, dried at 115° C. for 15 minutes, exposed to concentrated ammonium hydroxide vapor for 15 minutes, and scanned with the Photovolt Densitometer Model 525 (using a 505-millimicron broad band filter) coupled to the Varicord Variable-Response Recorder. For glycoprotein identification, the periodic acid-fuchsin sulfite method of Köiw and Gronwall (1952), as described by Durrum (1958), was used. The strips were scanned as described above.

Results

A. *Developmental stage extracts*

The brom phenol blue protein pattern obtained with unfertilized egg extracts is shown in Figure 1. Seven cation-bands and two anion-bands were noted. We chose to use the term bands rather than proteins since the absence of additional data such as electrophoresis at pH's other than 8.6, salt fractionation, etc., did not allow us to conclude that each band represented a single protein. Indeed, careful examination of the densitometer records often revealed that a major band was composed of two or more small peaks. For example, the cation-band labelled B in Figure 1 was actually composed of three smaller peaks, readily reproducible from run to run. In addition to these stained bands there was an additional band, labelled *fl*, which was not stained by brom phenol blue but which charred as a result of drying at 115° C. for 30 minutes and appeared as a yellow-brown band in the unstained strip. It was the only band which fluoresced under ultraviolet illumination. It did not correspond to any of the brom phenol blue-stained bands

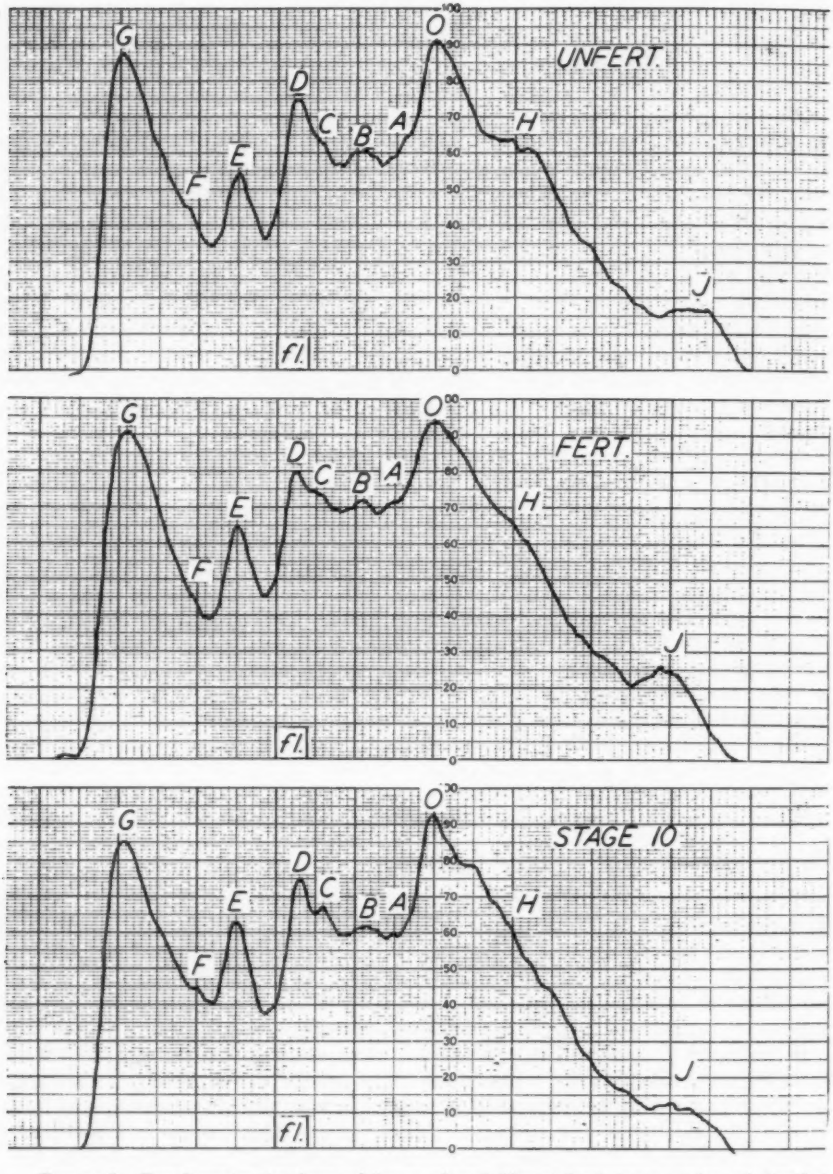

FIGURE 1. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Ordinate labelled 0 represents point of application. Values 0–100 on the 0 ordinate represent the relative density of brom phenol blue stain. To the right of the 0 ordinate are the anions; to the left are the cations. Abscissa units represent relative distance away from the point of application.

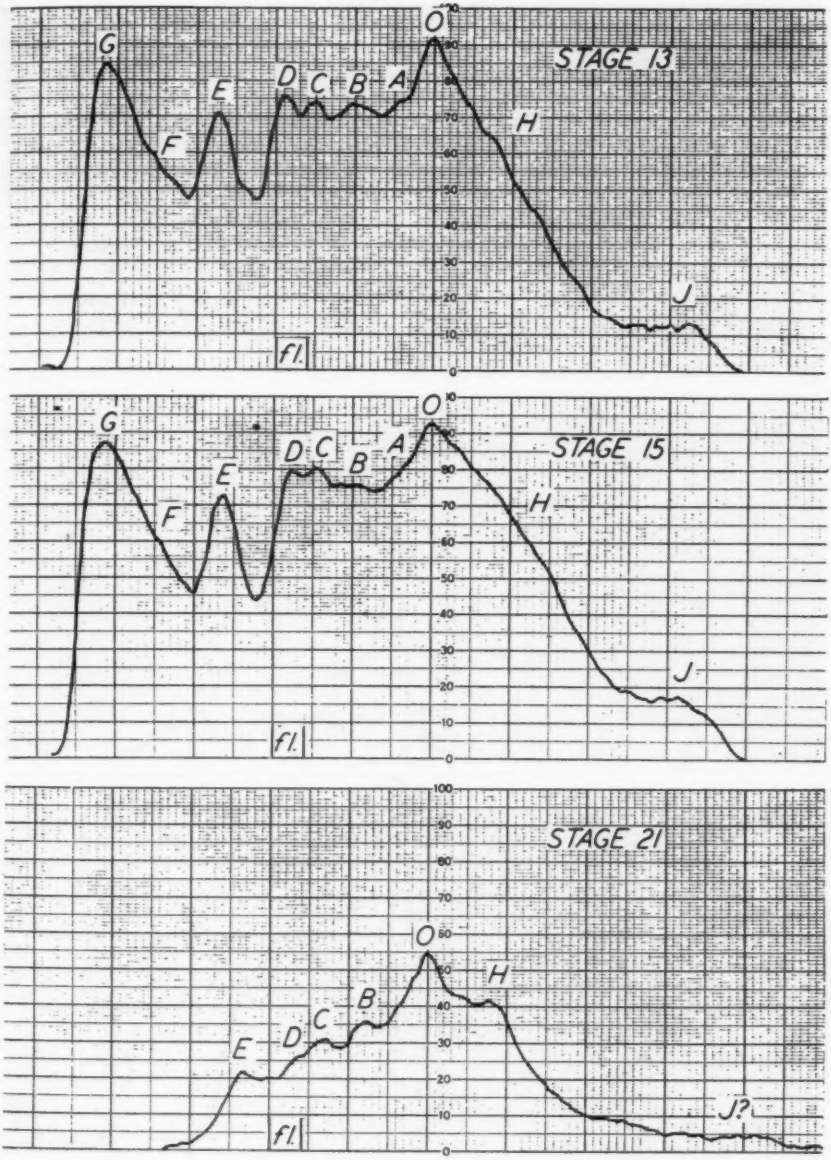

FIGURE 2. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Symbols as in Figure 1.

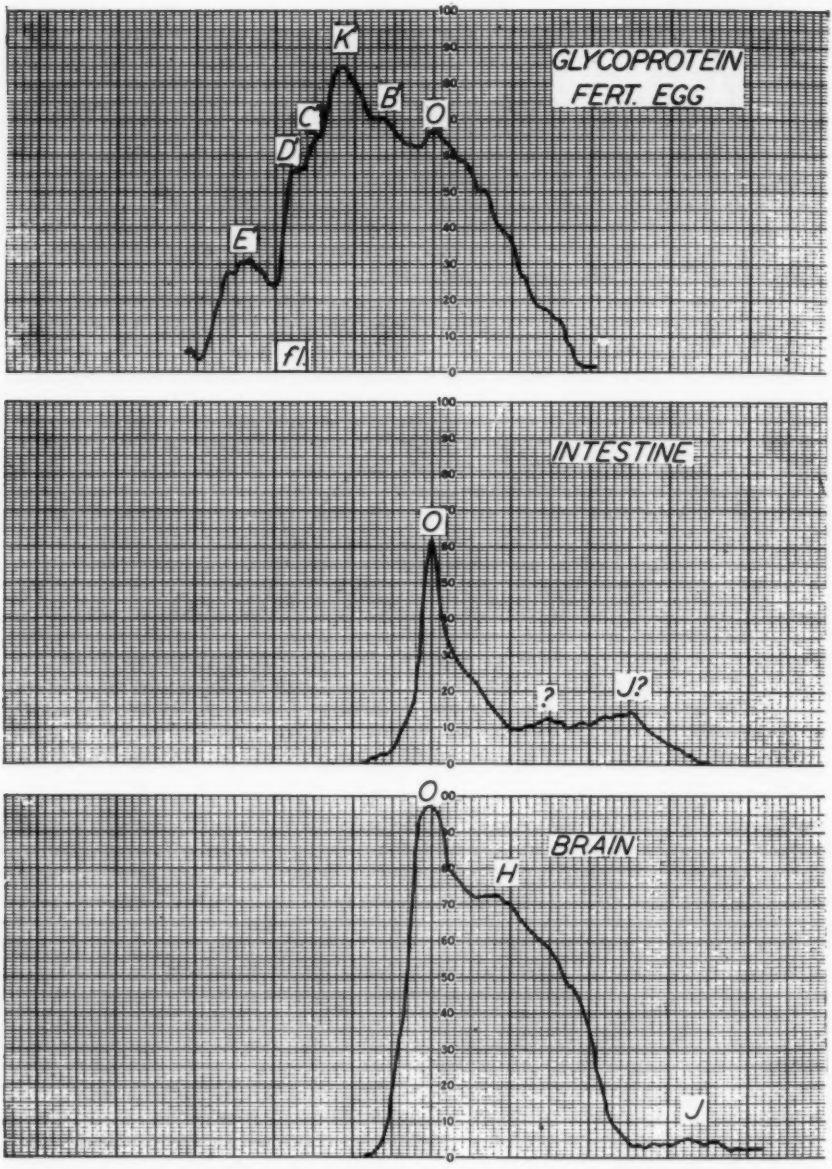

FIGURE 3. Upper: Densitometer tracings of periodic acid-fuchsin sulfite-stained paper strips of developmental stage extracts after electrophoresis. Middle and bottom: Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

and its limits were demarcated in the figure by the two lines on either side of the label *fl* in the figures.

The patterns obtained with fertilized egg extracts and stage 10 embryo extracts are shown in Figure 1. It was readily noted that following fertilization there was a decrease in the concentration of the H band which was perhaps comparable to the "Mirsky protein" found in the sea urchin egg. There was also a quite noticeable progressive increase during development in the concentration of the cation-band C. All other bands remained essentially constant in concentration through stage 15 (Figs. 1 and 2).

By stage 21, bands A, F and G were no longer detected (Fig. 2). The presence of band J was questionable and at best was in very low concentration and migrated as a broad band rather than as a sharply defined entity. The over-all protein concentration, under the conditions of extraction and electrophoresis employed, was markedly reduced. Band H, however, increased in relative concentration and by stage 21 approximated the relative concentration found in the unfertilized egg.

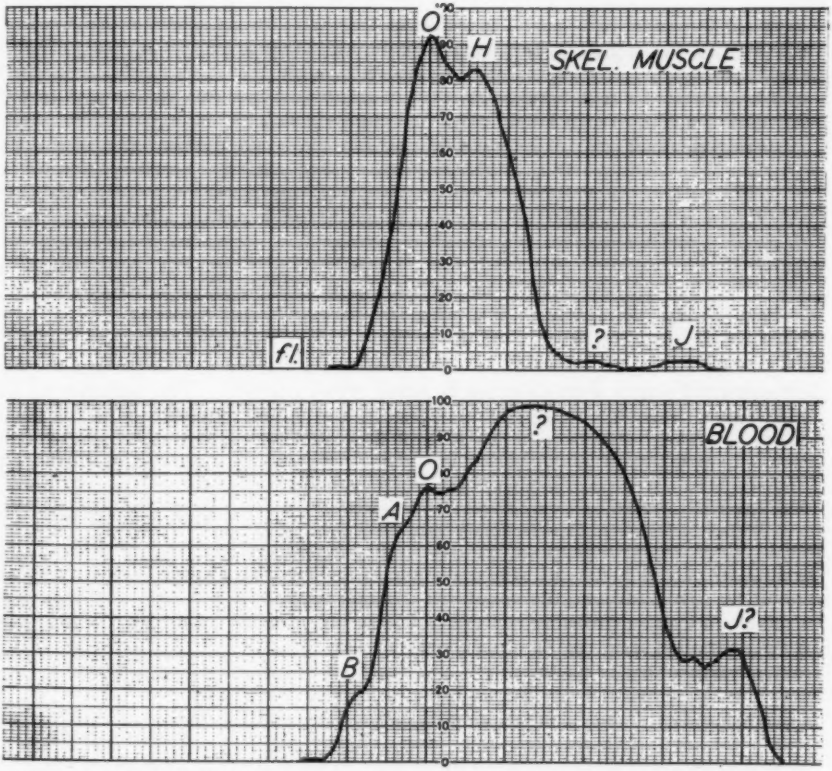

FIGURE 4. Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

Staining with the periodic acid-fuchsin sulfite method for glycoproteins revealed the presence of 5 stained bands, all migrating as cations (Fig. 3) in the fertilized egg extract (two hours after fertilization). One of these bands, K′, did not correspond to any of the brom phenol blue-stained bands. The remaining bands, B′, C′, D′ and E,′ corresponded in position to the B, C, D and E bands, respectively, of the brom phenol blue-stained strips.

The periodic acid-fuchsin sulfite pattern of the unfertilized egg extract was identical with the above results. Through stage 15, however, there was a progressive decrease in the stainability or concentration of these bands and by stage 21, the presence of carbohydrate was no longer detected. Reasons for labelling these bands with primes are considered in the discussion section.

B. *Adult organ extracts*

Patterns obtained with extracts of adult organs are demonstrated in Figures 3 and 4. These patterns revealed that relatively few of the ion-bands present in

TABLE I

Brom phenol blue-stained proteins of adult organ extracts present in unfertilized egg extracts

Adult organ extract	Band				
	fl	B	A	H	J
Intestine	−	−	−	−	±
Brain	−	−	−	+	+
Skeletal muscle	+	−	−	+	+
Blood	−	+	+	−	±

+ Indicates presence of band.
− Indicates absence of band.

developmental stages were detected in adult organ extracts. The majority of the cation-bands were not detected; in intestine and brain extracts, none were detected; in skeletal muscle only the *fl* band was detected; in the blood extract only the A and B bands were present. All of the anion-bands were detected, although not all were present in each adult organ extract.

Bands labelled with a question mark in the figures do not necessarily represent new or different proteins but rather represent pre-existing proteins which were unmasked by the changes in concentration of substances having almost identical mobilities. In addition, the anion-band labelled with a question mark present in the blood extract probably represents the serum proteins masked by hemoglobin. In the unstained strip the band was red in color.

The results obtained with adult organ extracts are summarized in Table I.

DISCUSSION

It is apparent from the results summarized in Table I that although the majority of the proteins extracted from adult organs are present in the unfertilized egg, the

protein patterns of adult organ extracts do exhibit differences. There are some components present in more than a single organ but others are apparently confined to a single organ. For example, bands H and J are present in both skeletal muscle and in brain extracts; the *fl* band, on the other hand, is present only in the skeletal muscle extract.

These patterns suggest that all, or most, of the proteins found in adult organs are first found in the unfertilized egg. As development proceeds some of the cells lose their ability to manufacture one or more particular proteins. Correlated with this loss of ability to synthesize a particular protein is a corresponding morphological differentiation. Differentiation, therefore, could involve a loss of the ability to synthesize a protein rather than the synthesis of a new protein. The work of Ebert (1953, 1955) on the distribution of cardiac myosin in the chick embryo is in support of this hypothesis. Ebert demonstrated that early in development cardiac myosin is rather uniformly distributed throughout the blastoderm. As development proceeds, the ability to synthesize cardiac myosin is eventually restricted to those cells located in the two heart-forming regions.

An alternative to the above explanation is equally probable. The differences observed in the protein patterns of adult organs and those of the embryo may reflect changes in the solubility of the proteins of the unfertilized egg or of adult organs rather than the loss or gain of any synthetic capacity. As a result of this line of reasoning, one could assume that the same proteins are present in the adult brain as in the unfertilized egg, but some of these proteins are insoluble, perhaps under the conditions of extraction, in the adult organ. At present, the more probable hypothesis cannot be determined. It is likely that both processes are involved in differentiation. The solubility and location of the proteins detected in this investigation are unknown. Whether we are dealing with the soluble proteins of the cytoplasm or those proteins soluble in the small amount of 10% Holtfreter's solution (and ultimately in the electrophoretic buffer) used for extraction is not readily apparent. Further studies are necessary to determine location, solubilities, etc. of these proteins. Why the present work does not show the appearance of "new" proteins (proteins insoluble in the unfertilized egg but soluble at later stages)—if the latter hypothesis is correct—cannot be answered at the present time.

It must be emphasized that the identification of proteins in this study has been made solely on the basis of mobility in an electric field. It is possible that new and different proteins from those of the unfertilized egg are being synthesized throughout development. These new proteins could have the same or closely matching mobilities as those of the early embryo, would therefore not be detected as new proteins, and could perhaps give the impression of a static population of protein types which does not exist. Further characterization of these proteins by other biochemical techniques will resolve this question. Of interest is the observation that, under these conditions, the majority of the embryonic proteins are cations; those of the adult are primarily anions. It is possible that those cation-bands which are lost during development represent the yolk proteins and are utilized as an energy source.

If the over-all hypothesis be true, that differentiation can be brought about by, or is associated with, the loss of the ability to synthesize a pre-existing protein

and/or changes in solubilities of pre-existing proteins, one must consider the ever-increasing body of literature apparently demonstrating the presence of new antigens in development. All of these papers, to our knowledge, have dealt solely with saline-soluble antigens. The appearance of a new antigen in development may rather represent a change in solubility rather than a synthesis of new antigenic material. For example, it is entirely possible that an antigen present in the blastula stage is saline-insoluble but in the gastrula stage is soluble in saline. Such an antigen, after the usual absorptions, etc. have been carried out, would then be described as a new antigen arising during gastrulation rather than as a change in solubility of a pre-existing substance. The demonstration by Markert and Møller (1959) of multiple forms of enzymes—the isozymes—which are tissue, ontogenetic, and species-specific can also be explained, rather simply, on the basis of changes in solubility.

The recent paper by Solomon (1959) offers a striking demonstration of changes in location, and therefore of solubility, of a particular enzyme during development. It was shown that the mitochondrial glutamic dehydrogenase of the chick embryo liver increased in activity after the twelfth day of incubation and this rise was followed by a sudden drop of glutamic dehydrogenase activity in the supernatant fluid (0.25 *M* sucrose) after 15 days of incubation. Supernatant fluid glutamic dehydrogenase activity is four times that of mitochondria at 7 days' incubation; 6 times at 12 days' incubation; ⅓ times at 20 days' incubation. The thesis that changes in the intra-cellular distribution of enzymes may occur during differentiation has been put forth by Krahl (1950), and Gustafson (1954).

The work of Schechtman (1955) and Nace (1953) has further demonstrated that the serum proteins of the chick are present in the unincubated egg and are probably transferred from the maternal circulation to the egg.

The results obtained using the periodic acid-fuchsin sulfite staining technique for glycoproteins remain to be considered (Fig. 3). The band K′ does not correspond to any of the brom phenol blue-stained bands and is probably a polysaccharide. The fact that the remaining four cation-bands, B′, C′, D′ and E′, correspond in position to the B, C, D and E bands, respectively of the brom phenol blue-stained strips, coupled with the observation that the periodic acid-fuchsin sulfite-stained bands were detected in early stages but not in stage 21 extracts, has led to the following interpretation.

It is possible that these bands represent a polysaccharide migrating at the same rate as proteins composing the band, or they may represent a glycoprotein. By stage 21, synthesis of these polysaccharides or glycoproteins has ceased or their solubility has changed. It would therefore be justifiable to label these bands as distinct from other proteins present in the band. It is also possible that the periodic acid-fuchsin sulfite technique is less sensitive than the brom phenol blue technique. Due to the decrease in protein concentration of the stage 21 extracts (Fig. 2) a positive brom phenol blue test but negative polysaccharide test results. Which of these interpretations is correct remains to be determined. The labelling of these bands with prime letters indicates the possibility of their being discrete entities.

It is realized that the present paper by no means offers conclusive proof, but merely suggests that differentiation may, in part, involve the loss of ability to synthesize a particular protein and/or changes in the solubility of pre-existing

proteins. The intra-cellular locations, concentrations, tissue distributions, and further biochemical characterizations of these proteins are being carried out in this laboratory at the present time.

Summary

1. Extracts of developmental stages and of four adult organs of the frog, *Rana pipiens*, were examined by the technique of paper electrophoresis.

2. Seven protein cation-bands and two protein anion-bands stained with brom phenol blue were detected in extracts of developmental stages from the unfertilized egg through stage 15. By stage 21, two of the cation-bands were no longer detected.

3. Examination of the adult organ extracts revealed organ-specific proteins in addition to proteins common to all organs tested.

4. The results were interpreted in terms of the hypothesis that differentiation involves, in part, the loss of ability to synthesize a particular protein(s) and/or changes in the solubility of pre-existing proteins.

Literature Cited

Clayton, R. M., 1953. Distribution of antigens in the developing newt embryo. *J. Embryol. Exp. Morphol.*, **1**: 25–42.

Cooper, R. S., 1946. Adult antigens (or specific combining groups) in the egg, embryo, and larva of the frog. *J. Exp. Zool.*, **101**: 143–172.

Cooper, R. S., 1948. A study of frog egg antigens with serum-like reactive groups. *J. Exp. Zool.*, **107**: 397–437.

Cooper, R. S., 1950. Antigens of frog embryos and of adult frog serum studied by diffusion of antigens into agar columns containing antisera. *J. Exp. Zool.*, **114**: 403–420.

Durrum, E. L., 1958. Paper electrophoresis. *In:* A Manual of Paper Chromatography and Paper Electrophoresis, by R. J. Block, E. L. Durrum and G. Zweig. Academic Press Inc., Publishers, New York, N. Y. Pages 489–667.

Ebert, J. D., 1951. Ontogenetic change in the antigenic specificity of the chick spleen. *Physiol. Zöol.*, **24**: 20–41.

Ebert, J. D., 1953. An analysis of the synthesis and distribution of the contractile protein, myosin, in the development of the heart. *Proc. Nat. Acad. Sci.*, **39**: 333–344.

Ebert, J. D., 1955. Some aspects of protein biosynthesis in development. *In:* Aspects of Synthesis and Order in Growth, D. Rudnick, editor. Princeton University Press, Princeton, N. J. Pages 69–112.

Flickinger, R. A., and G. W. Nace, 1952. An investigation of proteins during the development of the amphibian embryo. *Exp. Cell Res.*, **3**: 393–405.

Flickinger, R. A., E. Levi and A. E. Smith, 1955. Some serological experiments relating to the embryonic development of the lens. *Physiol. Zoöl.*, **28**: 79–85.

Gustafson, T., 1954. Enzymatic aspects of embryonic differentiation. *Intern. Rev. Cytol.*, **3**: 277–327.

Harding, C. V., D. Harding and P. Perlmann, 1954. Antigens in sea urchin hybrid embryos. *Exp. Cell Res.*, **6**: 202–210.

Harding, C. V., D. Harding and I. W. Bamberger, 1955. On cross fertilization and the analysis of hybrid embryonic development in echinoderms. *Exp. Cell Res.*, suppl., **3**: 181–187.

Köiw, E., and A. Gronwall, 1952. Staining of protein-bound carbohydrates after electrophoresis of serum on filter paper. *Scand. J. Clin. and Lab. Invest.*, **4**: 244–246.

Krahl, M. E., 1950. Metabolic activities and cleavage of eggs of the sea urchin, *Arbacia punctulata*. *Biol. Bull.*, **98**: 175–217.

Markert, C. L., and F. Møller, 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Nat. Acad. Sci.*, **45**: 753–763.

MIRSKY, A. E., 1936. Protein coagulation as a result of fertilization. *Science*, **84**: 333–334.

MONROY, A., 1950. A preliminary electrophoretic analysis of proteins and protein fractions in sea urchin eggs and their changes on fertilization. *Exp. Cell Res.*, **1**: 92–104.

NACE, G. W., 1953. Serological studies of the blood of the developing chick embryo. *J. Exp. Zool.*, **122**: 423–448.

NACE, G. W., 1955. Development in the presence of antibodies. *Annals N. Y. Acad. Sci.*, **60**: 1038–1055.

PERLMANN, P., AND T. GUSTAFSON, 1948. Antigens in the egg and early developmental stages of the sea urchin. *Experientia*, **4**: 481–482.

PERLMANN, P., 1953. Soluble antigens in sea urchin gametes and developmental stages. *Exp. Cell Res.*, **5**: 394–399.

SCHECHTMAN, A. M., 1955. Ontogeny of the blood and related antigens and their significance for the theory of differentiation. *In:* Biological Specificity and Growth, E. G. Butler, editor. Princeton University Press, Princeton, N. J. Pages 3–31.

SHUMWAY, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, **78**: 139–147.

SOLOMON, J. B., 1959. Changes in the distribution of glutamic, lactic and malic dehydrogenases in liver cell fractions during development of the chick embryo. *Dev. Biology*, **1**: 182–198.

SPAR, I., 1953. Antigenic differences among early developmental stages of *Rana pipiens*. *J. Exp. Zool.*, **123**: 467–497.

SPIEGEL, M., 1951. A method for the removal of the jelly and vitelline membrane of the egg of *Rana pipiens*. *Anat. Rec.*, **111**: 544.

SPIEGEL, M., 1955. The reaggregation of dissociated sponge cells. *Annals N. Y. Acad. Sci.*, **60**: 1056–1078.

TEN CATE, G., AND W. S. VAN DOORENMAALEN, 1950. Analysis of the development of the eye-lens in chicken and frog embryos by means of the precipitin reaction. *Konink. Med. Akad. Wetenschap.*, **53**: 894–909.

TYLER, A., 1957. Immunological studies of early development. *In:* The Beginnings of Embryonic Development. Amer. Assoc. Adv. of Sci., Washington, D. C. Pages 341–382.

TYLER, A., AND J. W. BROOKBANK, 1956. Antisera that block cell division in developing eggs of sea urchins. *Proc. Nat. Acad. Sci.*, **42**: 304–308.

TISSUE SPECIFICITY IN EMBRYONIC AND ADULT CYMATOGASTER AGGREGATA STUDIED BY SCALE TRANSPLANTATION

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The method of scale transplantation first used by Mori (1931) has proved to be of great value in studies on tissue specificity in fishes. Goodrich and Nichols (1933) and Hildemann (1956, 1957a, 1957b) have shown that, whereas autografts are always successful, homografts are invariably rejected by the host. These studies have established that the immune responses to homografts so profusely studied in mammals and birds are also observable in the goldfish (*Carassius auratus*).

The purpose of this investigation was to extend our knowledge of homograft reactions in fishes. Answers were sought to the following questions: (1) To what extent are adults of *Cymatogaster aggregata* (Gibbons) immunologically reactive toward scale homografts? (2) Are adults of this species capable of being sensitized to scale homografts (*i.e.*, will secondary, tertiary, etc., grafts be more rapidly rejected than the primary graft)? (3) Does the reactivity of embryonic and immature *C. aggregata* toward scale homografts differ from that of the adults? (4) Assuming affirmative answers to the above questions, can a sensitized, pregnant female transfer this homograft sensitivity to intra-ovarian embryos?

Materials and Methods

Experimental animals

C. aggregata is a marine embiotocid fish which is distributed over the west coast of North America from southern Alaska through Baja California (Tarp, 1952). Like all embiotocids this species is viviparous, the young spending about five months in the hollow ovary and increasing in size from 0.25 mm. (fertilized egg) to about 42 mm. (newborn) in standard length (Eigenmann, 1889; Triplett, unpublished data). Animals used in this investigation were collected by angling from Goleta pier, Goleta, California.

Methods

The method of grafting was essentially similar to the one devised by Hildemann (1957b). Adult fish were anesthetized in tricaine methane sulfonate (MS 222), 1/5000 dissolved in one per cent sodium chloride (MS 222 forms a white precipitate in sea water). Survival was greatest when the anesthetic was maintained at about 10° C. Grafting was done simply by removing a scale from the ventral surface of the host and replacing it with a scale from the dorsal surface of the

same fish or another fish. The transplant is easily distinguished since the scales of the dorsal surface of the body are heavily pigmented in contrast to those of the ventral surface which contain few or no melanophores. Grafted adults were maintained in aquaria provided with running sea water. They were fed *ad libitum* on pieces of mussel (*Mytilus californianus*).

Embryos were obtained by caesarian section using aseptic technique. They were then transferred to fingerbowls containing a modification (Triplett, unpublished data) of sterile White's medium (1954) and kept there until time was available for grafting. The embryos were anesthetized in 1/10,000 MS 222 dissolved in the aforementioned solution. An incision was then made in the flank skin with iridectomy scissors. Into this was placed either a homograft consisting of a sterile, adult, trimmed scale, or an autograft consisting of a piece of pigmented skin. The scales of embryos, when present, are too small and lightly pigmented to be used as autografts. Adult scales were sterilized by immersion for one minute in 0.004 *M* mercuric chloride dissolved in modified White's medium and washed with sterile medium before grafting. The scale remained alive and apparently healthy after such treatment. Embryos were grafted and maintained at 17° C. in a sterile culture chamber.

All host fishes, embryonic and adult, received a homograft and most fishes received an autograft. After it was established that autografts would survive and become healthy, some of the newborn in subsequent experiments were not given autografts in order to avoid this additional injury. In some cases reciprocal grafting of pairs of fishes was done. In others a single donor contributed scales to between three and eleven hosts.

The principle criterion for assaying the grafted scales was the condition of the scale melanophores. The melanophore is a single pigment cell of a radially dendritic pattern. The melanophores are usually somewhat separated from one another and readily visible under a dissecting microscope. These pigment cells contain many granules of melanin which respond to physiological changes to spread out into the cell's extremities, or to aggregate in the center of the cell. In a typical situation the melanophores of autografted scales, after an initial healing period, remained healthy and completely pigmented for an indefinite period of time. After a given time homograft melanophores began to undergo degenerative changes. The pigment aggregated as a black dot in the center of the melanophore and over a variable interval of time proceeded to fragment. The melanin granules were eventually phagocytized. In a few cases an inflammatory reaction similar to that described by Hildemann (1957b) and Goodrich and Nichols (1933) was observed. The time required for fragmentation of all the melanophores of the transplanted scale is here termed the reaction time.

Results

Adults

The adults of this species, like many pelagic fishes, are difficult to maintain in a laboratory without running sea water. Therefore the temperature, of necessity, was that of the ocean at that particular time of the year. In order to measure the effect of temperature on the reactivity of animals toward homografts one series was performed between March and July (average temperature = 17° C.),

and another series was done between August and October (average temperature = 20° C.).

In these series a single donor contributed scales to either three, four or five hosts. In all, 37 hosts and eight donors survived long enough to yield data. The hosts received as many as six successive grafts from the same donor, new grafts being placed as soon as fragmentation of the melanophores of the previous graft was complete. As mentioned above all the autografts placed on hosts remained alive and healthy for the duration of the experiment (in some cases more than three months).

Both autografts and homografts underwent an initial healing phase during which time the melanophores that had been injured during the operation fragmented and were phagocytized. After the third post-operative day degenerating melanophores could no longer be found in the autografts, but progressive loss of melanophores continued in the homografts until all had disintegrated (reaction time). The reaction time for homografts is shown in Table I. Here it can be seen that the average reaction time for grafts at 17° C. is 7.0 days. The reaction time of subsequent grafts decreased progressively until at the fourth grafting it averaged 3.5 days. Any further grafting was not effective in increasing homograft sensitivity.

It can also be seen in Table I, in confirmation of Hildemann's (1957b) findings, that increased temperature of the external medium has the effect of decreasing the reaction time. It was also observed that at the higher temperature the primary homograft has a more pronounced effect in producing sensitivity to subsequent grafts. These observations are paralleled by those of Cushing (1942) who found that the ability of fishes to produce antibody against injected erythrocytes or spermatozoa is influenced by temperature in such a way that circulating antibody appears sooner at higher than at lower temperatures.

Some evidence has been gathered which indicates (as might be expected) that fishes of this species, comprising a natural population of unknown interrelationships, bear some but not all scale tissue factors (antigens?) in common. Six fish of the series discussed above, which had received between four and six successive homografts, were each given a graft from a donor that had not previously been used. The average reaction time for this series was 5.6 days. The value for a primary graft would have been 7.0 days (average), and the value for a fifth, sixth or seventh set reaction would have been 3.5 days (average). Since the realized value is in between these two, the statement that each fish bears distinctive as well as common homograft factors seems justified. Medawar (1946) has made similar observations on rabbits.

Significance of reaction time

A small series of operations was performed in an effort to determine the relationship of the reaction time (melanophore disintegration time) to the complete destruction of the living tissue of the homograft. The method, first used by Hildemann (1957b), consisted of returning homografts to the donor after variable lengths of time. Failure of the graft to re-establish itself in the original donor indicated irreparable damage or death.

In this series five pairs of adult fish were used. Each donor contributed 10

scales to its partner, and the grafted scales were returned to the donor at daily intervals. It was found in all five cases that scales returned to the host on or before the third day after the original grafting became re-established in the donor. Those returned on or after the fourth day did not survive. These data lack statistical

TABLE I

Reaction time in days for scale grafts to adult fish

Operations performed between March and July—Average water temperature 17° C.

Specimen no.	1st graft	2nd graft	3rd graft	4th graft	5th graft	6th graft
1-1	11					
2	7	7				
3	9	9	6	3	3	3
4	?	8	3	3	3	4
5	—	4				
2-1	9	11				
2	11	11				
3	11	11				
4	11	9				
5	3	10	4	4	3	
3-1	7	9				
2	6					
3	6	6	4	3		
4	6	5	6	5		
5	9	4				
4-1	11					
2	6	5				
3	11	6	3	3	4	
4	11	5	6	3	4	3
5	11	5	6	4	4	
5-1	6	7	4			
2	7	7				
3	6	7	3			
4	6	8	4			
5	6	5	5			
Average	7.0	7.2	4.5	3.5	3.5	3.3
Operations performed between August and October—Average water temperature 20° C.						
1B-1	5					
2	5					
3	5	3				
4	7					
2B-1	5	3				
2	7	3				
3	5					
4	5	3				
5	7					
4B-1	7	3				
2	7	3				
3	5	3				
Average	5.8	4.0				

force but suggest that homografts are no longer viable in a suitable environment several days before the reaction time (7.0 days at this temperature). It is felt, however, that the reaction time as used in the various experiments reported here is valid since all other data indicate that it is a function of host homograft sensitivity.

Embryos

Two series of experiments were done on intra-ovarian embryos. The hosts of the first series consisted of six siblings averaging 29 mm. standard length, and the hosts of the second series consisted of 11 siblings averaging 19 mm. standard length. The hosts in each series received homografts from a single adult, the operation and postoperative care being done in the manner described above.

Since the grafts had to be examined every day, and since the medium was rich in organic materials some difficulty was experienced in maintaining asepsis. In the first series, four animals lived 16 days, one lived 20 days and one lived 24 days. In the second series eight animals lived 20 days, and three lived 30 days.

An initial healing period lasted between three and five days. During this time damaged melanophores of both autografts and homografts were phagocytized. After this time both autografts and homografts remained alive and in excellent condition as long as the host lived. Since some of the embryos lived as long as 30 days there is a strong indication that intra-ovarian animals are incapable of eliciting the homograft reaction.

Immature fishes

The animals used in this series were divided into two groups on the basis of their parturition age. There survived in the first group 11 animals grafted one to three days after birth, and in the second group seven fishes grafted 15 days after birth. Some, but not all animals received an autograft consisting of a 0.25-mm. piece of skin, and all received a scale homograft from an adult fish. Each donor contributed to between three and seven hosts. All animals in this series were cultured at approximately 17° C. in running sea water.

The autografts remained alive for the duration of the experiment. The homografts were without exception broken down in a manner similar to that described for the adult host homografts. It can be seen in the first two columns of Table II that the reaction time for homografts is approximately in inverse proportion to the age of the fish. The average reaction time for one- to three-day fishes is 13.2 days, whereas that for 15-day fishes is 9.7 days (that for adults is 7.0 days at the same temperature).

Embryos from sensitized parents

These experiments were performed in an effort to discover whether or not induced homograft sensitivity in parent fishes could be transferred to intra-ovarian embryos. A total of 11 pregnant females was sensitized. Ten were given a single scale homograft and one was given three successive homografts. The young were born from 10 to 30 days after the parent had received the last graft. The newborn animals each received a scale homograft one to three days after birth

TABLE II

Host aged 1–3 days		Host aged 15 days		Host aged 1–3 days		Host aged 1–3 days	
Post-partum. Parent not sensitized.		Post-partum. Parent not sensitized.		Post-partum. Parent given 1 or 2 successive homografts.		Post-partum. Parent given 3 successive homografts.	
Specimen no.	Reaction time	Specimen no.	Reaction time	Specimen no.	Reaction time	Specimen no.	Reaction time
S-6-59-1a	16	S-6-59-7a	8	S-8-59-1a	7	S-8-59-19a	7
1b	16	7b	10	1b	7	19b	7
1c	16	7c	10	1c	7	19c	7
S-6-59-2	5	7d	10	1d	9	19d	7
3	15	7e	10	1e	9	19e	7
4	12	7f	10	S-8-59-2	13	19f	9
6a	12	7g	10	S-8-59-7	6	19g	9
6b	12			S-8-59-9a	3	19h	9
6c	12			9b	9	19i	11
6d	12			S-8-59-10a	9		
6e	17			10b	11		
				S-8-59-13a	8		
				13b	8		
				13c	10		
				S-8-59-14a	8		
				14b	11		
				14c	11		
				14d	11		
				14e	11		
				S-8-59-18a	8		
				18b	8		
				18c	10		
				18d	10		
				18e	10		
				S-8-59-21a	8		
				21b	8		
				21c	8		
				21d	11		
				S-8-59-22a	4		
				22b	6		
Average reaction time	13.2	9.7		8.6		8.1	
Total no. specimens	11	7		30		9	

from the same donor that had been used for the parent. The results are given in the third and fourth columns of Table II. The results for the progeny of singly grafted parents are tabulated separately from those for the progeny of the triply grafted parent. By comparing column one with columns three and four it can be seen that the reaction time for one- to three-day young from sensitized parents (average reaction time = 8.6 and 8.1 days) is significantly shorter than that for young of comparable age from unsensitized parents (average reaction time = 13.2

days). It can also be seen by comparing columns three and four that the parent with three successive grafts produced young with slightly greater sensitivity (average reaction time = 8.1 days) than did the parents with a single graft (average reaction time = 8.6 days). The evidence leaves little grounds for doubt that homograft sensitized parents can transfer this sensitivity to intra-ovarian young.

Discussion

These experiments indicate a basic similarity between adult *C. aggregata* and *Carassius auratus* (Hildemann, 1957b) concerning the homograft response. Both species reject homografts, and both can be sensitized by successive grafting. In both species the time for homograft rejection is shortened by increase in temperature. Barrymore (unpublished data) has made similar observations on adult swordtails (*Xiphophorus helleri*) as have Triplett and Barrymore (unpublished data) on *Amphistichus argenteus* and *Hyperprosopon argenteum*. Fin transplantations on xiphophorin fishes yield similar results (Kallman and Gordon, 1957). There is good reason to suspect that these phenomena could be observed in teleosts in general.

To the authors' knowledge only one publication to date has dealt with tissue transplantation in older embryonic or immature fishes. Humm *et al.* (1957) have transplanted melanomas from adult platyfish-swordtail hybrids to embryos of both species and their hybrids. These workers have found, in contrast to the present authors, that homografts on embryonic fishes will not survive. This difference in results could be explained in one or both of two ways. First, it should be noted that the tissues grafted by Humm and his colleagues were not normal and should not be expected to behave as such. The inability of the transplanted melanomas to survive could be interpreted as a physiological deficiency other than immunological incompatibility.

A second and more likely possible explanation must take into account the different developmental rates of platyfishes and swordtails as compared with *C. aggregata*. The young of the former two species become free-living in about three weeks (Hooper, 1943), whereas the latter spends about five months in the ovarian cavity of the parent. A close correlation has been noted between the time of parturition in mammals (*e.g.*, Freund, 1930), hatching in birds (*e.g.*, Murphy, 1914; Waterman, 1936) or metamorphosis in amphibians (Schwind, 1933, 1937; Triplett, 1958) and the development of the ability to react immunologically to implanted or injected foreign materials. If our interpretation of Humm's work is correct those animals that were not killed by mechanical blockage, resulting from a rapidly proliferating graft, retained the transplant until well after the normal time of birth. The present experiments show that in *C. aggregata* homograft incompatibility is expressed soon after birth. This is quite possibly also the case in xiphophorin fishes used by Humm *et al.*

There has been considerable speculation as to whether or not circulating antibody is responsible for tissue incompatibility (reviewed by Snell, 1957). The first clear proof of an immune reaction to homografts was published by Gorer (1937) who found that tumor homografts could elicit the formation of circulating antibodies capable of agglutinating donor red cells. More recent work (see Snell, 1957) has established that humoral antibodies are formed in a variety of species

against a variety of transplanted tissues. Santos *et al.* (1959) have shown that the serum of graft-sensitized rodents, upon injection into other animals, can produce a passive heterograft sensitivity.

The present experiments on transfer of homograft sensitivity from parent to intra-ovarian embryos can be explained best by assuming that circulating antibody is produced against scale homografts. It is probable that antibody accumulates in the ovarian fluid and is ingested by the embryos (no placental connection exists). This hypothesized method of transfer is made more plausible by the fact that Hemmings and Morris (1959) have been able to observe the absorption of antibody from the gut in young mice. In addition, Brambel *et al.* (1959) have observed that homologous antitoxin is absorbed from the uterine cavity to the fetal circulation in the rabbit.

Hosoda *et al.* (1955) have reported experiments of interest in this context. They found that hens injected with horse erythrocytes produce antibodies and transfer these antibodies to offspring.

Summary

1. Embryonic and adult *Cymatogaster aggregata* have been subjected to scale homotransplantations.

2. Homografts are rejected after a short period of time. The time required for graft rejection is temperature-dependent.

3. Increased sensitivity to homografts results from successive transplantations.

4. Intra-ovarian embryos used in these experiments were not capable of rejecting scale homografts.

5. Immature free-living fish rejected homografts, but more slowly than adults.

6. Pregnant females can transfer homograft sensitivity to intra-ovarian embryos. It is hypothesized that circulating antibody is passed to the embryo via the ovarian fluid and the embryonic hindgut.

LITERATURE CITED

Brambel, F. W. R., W. A. Hemmings and C. L. Oakley, 1959. The relative transmission of natural and pepsin-refined homologous antitoxin from the uterine cavity to the fetal circulation in the rabbit. *Proc. Roy. Soc. London, Ser. B*, **150**: 312–317.

Cushing, J. E., 1942. An effect of temperature upon antibody-production in fish. *J. Immun.*, **45**: 123–126.

Eigenmann, Carl H., 1889. On the development of California food fishes. *Amer. Nat.*, **23**: 107–110.

Freund, J., 1930. Influence of age upon antibody production. *J. Immun.*, **18**: 315–324.

Goodrich, H. B., and Rowena Nichols, 1933. Scale transplantation in the goldfish, *Carassius auratus*. *Biol. Bull.*, **65**: 253–265.

Gorer, P. A., 1937. The genetic and antigenic basis of tumor transplantation. *J. Pathol. Bacteriol.*, **44**: 691–697.

Hemmings, W. A., and J. G. Morris, 1959. An attempt to affect the selective absorption of antibodies from the gut in young mice. *Proc. Roy. Soc. London, Ser. B*, **150**: 403–409.

Hildemann, W. H., 1956. Scale homotransplantation in goldfish (*Carassius auratus*). *Transpl. Bull.*, **3**: 67–68.

Hildemann, W. H., 1957a. Early onset of the homograft reaction. *Transpl. Bull.*, **3**: 144–145.

Hildemann, W. H., 1957b. Scale homotransplantation in goldfish (*Carassius auratus*). *Ann. New York Acad. Sci.*, **64**: 775–791.

HOOPER, A. F., 1943. The early embryology of *Platypoecilus maculatus*. *Copeia*, **4**: 218–224.

HOSODA, T., T. KANEKO, K. MOGI AND T. ABE, 1955. Transfer of antibodies in the hen to her eggs and to the offspring. *Bull. Natl. Inst. Agric. Sci. (Japan) Ser. G. Animal Husbandry*, **10**: 125–129.

HUMM, D. G., E. E. CLARK AND J. H. HUMM, 1957. Transplantation of melanophores from platy-swordtail hybrids into embryos of swordtails and platyfish and their hybrids. *J. Exp. Biol.*, **34**: 518–528.

KALLMAN, KLAUS D., AND MYRON GORDON, 1957. Transplantation of fins in Xiphophorin fishes. *Ann. New York Acad. Sci.*, **71**: 305–320.

MEDAWAR, P. B., 1946. Immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals. *Brit. J. Exp. Path.*, **27**: 9–14.

MORI, YASUMASA, 1931. On the transformation of ordinary scales into lateral scales in the goldfish. *J. Fac. Sci. Imp. Univ. Tokyo*, **2**: 185–194.

MURPHY, J. B., 1914. Studies on tissue specificity. II. The ultimate fate of mammalian tissues implanted in the chick embryo. *J. Exp. Med.*, **19**: 181–186.

SANTOS, GEORGE W., LEONARD J. COLE AND RICHARD M. GARVER, 1959. Antigenic stimuli for transplantation immunity to rat bone marrow heterografts in lethally x-irradiated mice. *J. Immunol.*, **83**: 66–73.

SCHWIND, JOSEPH L., 1933. Tissue specificity at the time of metamorphosis in frog larvae. *J. Exp. Zool.*, **66**: 1–14.

SCHWIND, JOSEPH L., 1937. Tissue reactions after homoplastic and heteroplastic transplantation of eyes in the anuran amphibia. *J. Exp. Zool.*, **77**: 87–107.

SNELL, C. D., 1957. The homograft reaction. *Ann. Rev. Microbiol.*, **11**: 439–458.

TARP, FRED H., 1952. A revision of the family Embiotocidae (the surfperches). *Fish Bulletin No. 88, State of Calif. Dept. of Fish and Game, Bureau of Marine Fisheries.* 1–99.

TRIPLETT, EDWARD L., 1958. The development of the sympathetic ganglia, sheath cells, and meninges in amphibians. *J. Exp. Zool.*, **138**: 283–312.

WATERMAN, A. J., 1936. Heteroplastic transplantation of embryonic tissues of rabbit and rat. *Amer. J. Anat.*, **60**: 1–25.

WHITE, PHILLIP R., 1954. The Cultivation of Animal and Plant Cells. The Ronald Press Co., New York, p. 90.

SOME ASPECTS OF OSMOREGULATION IN EMBRYONIC AND ADULT CYMATOGASTER AGGREGATA AND OTHER EMBIOTOCID FISHES

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The Embiotocidae is a family of marine (with one exception) teleosts which is somewhat unique in that all species possess a truly viviparous type of development. The young remain in the hollow ovary of the parent for a period of about five months, during which time they increase in size from 0.24 mm. (fertilized egg) to about 42 mm. (newborn) (Eigenmann, 1890, 1894). It was learned while devising a technique for culturing the embryos *in vitro* (Triplett, unpublished) that, unlike the adults, they are capable of tolerating only very limited variation in the osmotic concentration of the external medium.

The object of this study was to learn more precisely how well these animals can regulate to variations in the concentration of the external medium both as adults and during development. In addition, preliminary experiments were performed in an effort to find out why the adults are better able to regulate than the embryos.

Materials and Methods

Experimental animals

Cymatogaster aggregata (Gibbons) was the most easily obtained of the embiotocids and was therefore the principal organism used in the investigation. This species is widely distributed over the west coast of North America, ranging from southern Alaska through Baja California (Tarp, 1952). It has been observed in estuaries where the salinity is quite variable (Hubbs, 1917) as well as in bays and along the open coast. Animals used in these experiments were collected by angling from Goleta Pier, Goleta, California.

Less extensively investigated were *Amphistichus argenteus* (Agassiz) and *Micrometrus minimus* (Gibbons). These species have about the same distribution as *C. aggregata*, but so far as the authors are aware, they do not enter brackish water. For these experiments they were collected with a beach seine on the campus beach at Goleta, California. Only adults of these two species were used.

Methods

The general approach was to determine the total osmotic pressure of the serum and the change in body weight after exposure to experimental salinities for periods ranging from six hours to nine days.

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The experimental animals were divided into four groups on the basis of their stages of development. Group 1 comprised intra-ovarian embryos ranging in standard length from 18 mm. to 25 mm. At this stage the animals were completely transparent except for a thin band of melanophores along the lateral line and a few scattered melanophores in the head region. The fins were hypertrophied and possessed highly vascularized dermal flaps between the rays. The hindgut was also hypertrophied and protruded in the cloacal area. The fins and hindgut probably serve as a pseudo-placenta (Eigenmann, 1890, 1894). The trailing edge of the caudal fin was convex or flat. Group 2 consisted of intra-ovarian embryos measuring between 27 mm. and 31 mm. in standard length. These animals still possessed hypertrophied fins and a hypertrophied protruding hindgut. Melanin deposition had increased considerably, and guanophores appeared on the dorsal half of the animal, rendering it semi-opaque. Scales had differentiated visibly at this time. The trailing edge of the caudal fin was becoming concave. Group 3 comprised intra-ovarian animals nearing parturition age and ranging in standard length from 32 mm. to 38 mm. The body was completely opaque and the adult pigment pattern was acquired. The fins, though not yet of adult proportion, had begun to atrophy. The hindgut had also atrophied and protruded only slightly. The trailing edge of the caudal fin was deeply concave and V-shaped as in the adult. Group 4 comprised newborn animals and adults measuring between 39 mm. and 115 mm. standard length. Preliminary experiments on group 4 animals of different lengths (ages) established that their osmotic responses were identical.

A minimum of four animals from each group was placed directly from holding tanks (running sea water) or directly from the ovarian cavity into each of the following solutions of sea water diluted with distilled water: 100% ($\Delta = 1.86$), 75%, 50%, 25%, 20%, 15%. For each series of measurements four adult animals were kept in an aerated battery jar containing 3000 ml. of fluid and maintained at 16° C., or four embryos were kept aseptically in a fingerbowl containing 1000 ml. of fluid and maintained at the same temperature. Serum freezing point determinanations and weight measurements were made on four different animals of each group at each dilution after six hours and one, two, four and eight or nine days. Freezing points are here given in sodium chloride equivalents.

Weight determinations were made on blotted animals with a triple beam balance to an accuracy of 0.01 gram. All animals in group 4 were weighed before and after exposure to the experimental medium. Weight measurements were not possible with groups 1, 2, and 3 because these animals will not live after such handling.

Blood for freezing point determinations was taken by cardiac puncture or by removal of the tail at the level of the caudal peduncle. In the adults the particulate matter was removed before placing the serum in capillaries. This was not possible with the embryos since too little blood was available. Instead the freezing point capillary was inserted into the heart, and whole blood was drawn into the tube. The red cells and the clot were then packed by centrifuging, thus rendering the serum clear enough for freezing point determinations. Freezing points were determined with an apparatus modified after one used by Gross (1954), and since the modification offers some advantages over the original it is briefly described here. It consists of a Fiberglas-lined, insulated box containing four meters of one-half inch copper tubing. Two glass windows allow light to be passed through

the box. The box is filled with brine, and the brine is cooled by circulating freon-14 through the copper tubing. When the desired temperature is obtained the freon-14 is turned off, and the previously frozen capillaries containing the fluid to be measured are inserted in the brine between the observation windows. As the box warms very slowly the fluid in the capillaries melts. The melting point can be observed with great accuracy ($\pm$ 0.02° C.) by using crossed polaroids.

In an effort to learn something about differences in ionic exchange between different age groups preliminary experiments have been performed using chlorine-36 as a tracer. The technique consisted of placing the experimental animal, after peritoneal injection of radio-chloride, in a divided chamber so that the gills and half the body were on one side. Aliquots of the fluid in the two compartments were measured at intervals over a three- to eleven-hour period for radioactivity. The fluid in both compartments was a modified White's (1954) medium (.220 *M*/L NaCl equiv.) slightly hypertonic to the serum.

Results and Discussion

Changes in serum freezing point and body weight in experimental media

Cymatogaster aggregata: The results of freezing point determinations and weight measurements on adult (group 4) *C. aggregata* are summarized in Figure 1, each point on the graph representing the average of measurements made of at least four fishes. It can be seen that the viable range of salinities for this species is quite large, ranging in these experiments from 20 per cent to 100 per cent sea water. Adults remained alive no longer than three hours in 15 per cent sea water, during which time they increased 3.83 per cent in weight.

At all salinities below 100 per cent sea water there was an initial drop in serum tonicity which reached a minimal value between one and two days after exposure to the experimental media. Accompanying this was a corresponding increase in wet weight. In general, the minimal serum osmotic value decreased as the salinity of the external medium was decreased. Less convincingly, Figure 1 indicates that as the salinity of the external medium is decreased, the time required for return to normal weight and serum tonicity increases. At all salinities tested above 20 per cent sea water, regulation is complete or nearly complete within nine days.

It will be noted that there was a small temporary increase in serum tonicity and decrease in wet weight of fishes maintained in 100 per cent sea water. The authors attribute this to an increased permeability of the integument resulting from handling. It is presumed that a similar increase in integumentary permeability was also experienced by fishes kept in other sea water dilutions, and this must be kept in mind when interpreting the results.

The results of freezing point determinations on the various embryonic stages are summarized in Table I, and corresponding figures for the adults are included for comparison. It can be seen that the viable range of salinities for embryos increases with increase in age. Group 1 and group 2 were able to survive for the complete duration of the experiment only in media between 25 and 36 per cent sea water (36 per cent sea water is equivalent osmotically to the ovarian fluid in which the animals normally reside), whereas group 3 was capable of regulation at all salinities between 25 and 75 per cent sea water.

However, it was noted that, in general, it required a greater alteration of the

serum osmotic potential of embryos than of adults to kill the animals. In these experiments group 1 and group 2 animals died when this value was above .232 *M*/L or below .114 *M*/L. Group 3 animals died at values above .223 *M*/L or below .156 *M*/L. Possibly for this reason the embryos live longer at 15 per cent sea water than do the adults. This may be a reflection of the fact that less differentiated systems are generally more tolerant of their physiological environment.

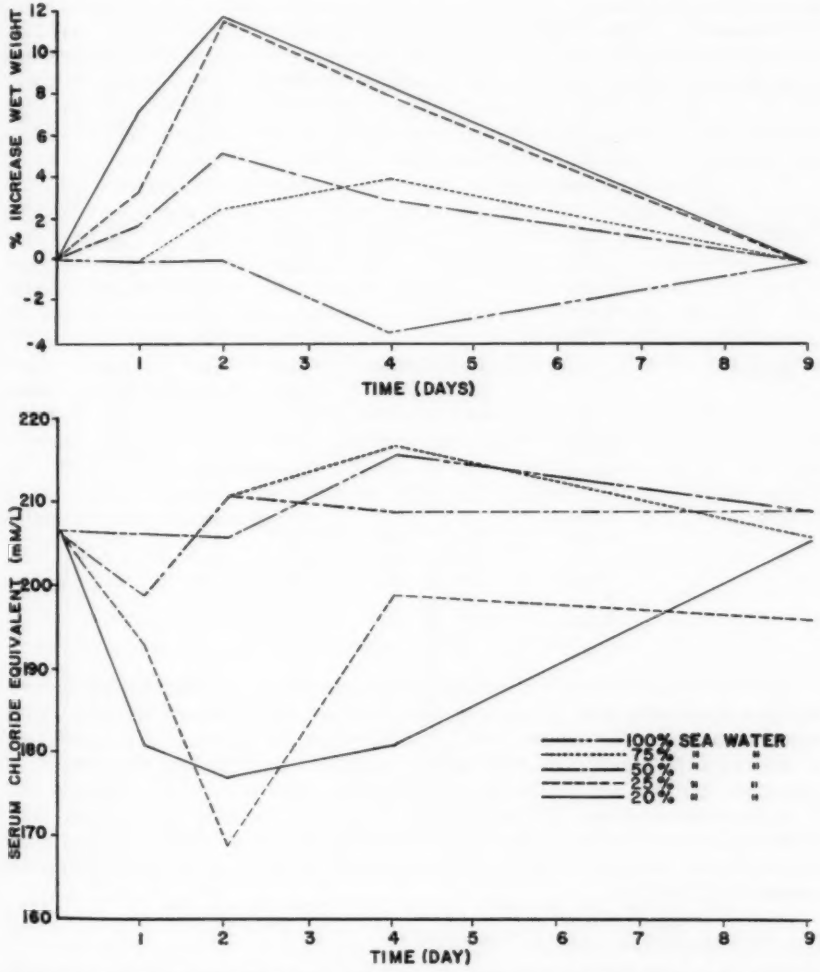

FIGURE 1. Upper graph: the wet weight of *C. aggregata* adults plotted as a function of time media of different salinities. Lower graph: tonicity (sodium chloride equivalents) of *C. aggregata* adult serum plotted as a function of time in media of different salinities.

TABLE I

Serum tonicity of C. aggregata of different developmental stages after exposure to media of variable salt concentrations for variable intervals of time

		External medium					
% Sea water NaCl equiv. (*M*/L)		100 .568	75 .326	50 .284	25 .142	20 .114	15 .085
Age group	Time exposed						
1. Transparent	6 hours	death	death	.230	.160	.135	.120
	1 day			death	.164	.131	.115
	2 days				.185	death	death
	4 days				.192		
	6 days				.190		
	8 days				.195		
2. Semi-opaque	6 hours	.232	.210	.205	.160	.135	.125
	1 day	death	death	.245	.156	.138	.114
	2 days			death	.181	.158	.125
	4 days				.185	death	death
	6 days				.190		
	8 days				.195		
3. Opaque	6 hours	.227	.198	.196	.192	.198	.179
	1 day	.233	.202	.209	.191	.197	.179
	2 days	death	.236	.221	?	?	death
	4 days		.221	.215	.195	.156	
	6 days		?	?	?	death	
	8 days		.211	.209	.206		
4. Adult	6 hours	?	?	?	?	?	death
	1 day	.206	.199	.193	.193	.181	
	2 days	.206	.211	.211	.169	.177	
	4 days	.216	.217	.209	.199	.181	
	6 days	?	?	?	?	?	
	9 days	.209	.206	.216	.196	.209	

Micrometrus minimus and *Amphistichus argenteus:* Similar though less extensive experiments were performed with the adults of these two species. It was expected that since they do not enter brackish water, the ability of *M. minimus* and *A. argenteus* to regulate to differentiate salinities would be less than that of *C. aggregata,* but this was not the case. Both were quite capable of remaining alive in media between 20 and 100 per cent sea water over the nine-day interval. Furthermore, serum freezing points of animals living in any of these dilutions at the end of this time were the same as those of animals living in the normal environment (serum = .209 *M*/L).

Ion exchange studies using Cl^{36} tracers

The decreased ability of embryos to regulate to different tonicities of the external medium as compared to adults could be explained in several ways. One possible explanation is that these animals may not yet have differentiated the

branchial salt secretory mechanism that has been demonstrated in various adult teleost fishes (Smith, 1930). Both adults and embryos of *C. aggregata* were examined for this mechanism by utilizing the divided box technique first used by Smith (1930). The animals were injected intraperitoneally with 0.25 ml. of an isotonic salt solution with an activity of 0.125 microcuries per milliliter. At two-hour intervals or less, 250-lambda samples were withdrawn from the anterior and from the posterior chambers and measured for radioactivity with an accuracy of 1.0 per cent.

Three adults ranging in standard length from 105 mm. to 115 mm. and three sibling embryos (group 3) measuring 33 mm. were used. The results for one animal of each group are shown in Figure 2. The general results were about the same for any animal in a given age group. It can be seen that in the adults the activity of the anterior chamber is consistently higher than that of the posterior for the duration of the experiment. This would indicate that a branchial salt secretory mechanism is operative. However, in the embryos, the activity of the posterior was consistently higher than that of the anterior chamber. This is tentatively interpreted to mean that the "salt pump" in the gills is not yet in operation or is operating at a much reduced rate. Though the embryos were placed in the chamber so that half the body was on each side, it was roughly estimated that 74 per cent of the body surface area was contained by the posterior chamber. The hypertrophied fins account for the greater part of this area. This could account for the higher Cl^{36} value in the rear chamber by assuming that chloride loss is about equal over the whole body surface (including the gills).

These tracer studies are quite obviously not sufficient to draw definite conclusions. The sample is small, and desirable control experiments have not been performed. The experiments do suggest, however, that the "salt pump" operative in the adult is absent or operating less efficiently in the embryo. Further efforts in this direction are planned.

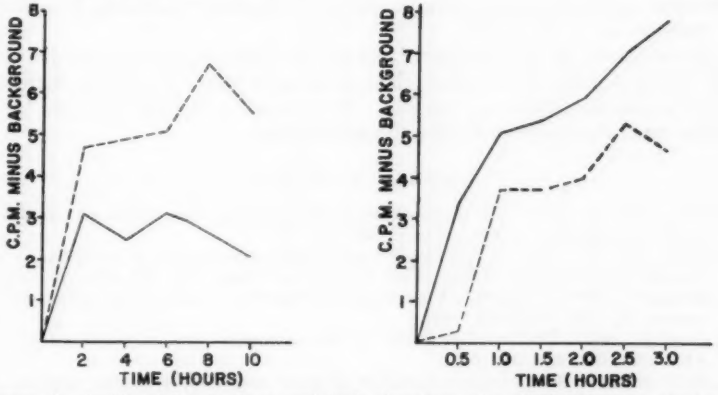

FIGURE 2. Radioactivity in front and back chambers of a divided box so arranged that the anterior half of a chloride^{36}-injected fish is contained in the front chamber and the posterior half of the fish is contained in the back chamber. Dotted lines = front chamber; solid lines = back chamber. Left graph = measurement for adult fish; right graph = measurements for embryonic fish.

Another clue concerning the lack of osmoregulatory ability in embryos was gained by observing the volume of the hindgut lumen in media of different osmotic concentrations. It was noted that embryos placed in any of the hypertonic solutions (> 36% sea water) experience a swelling of the hindgut. The hindguts of animals placed in hypotonic solutions shrink. It is well known that marine teleosts maintain osmotic equilibrium by drinking sea water and excreting the excess salt, principally through the gills. It is hypothesized that these embryos also drink the external medium. If they should be placed in a hypertonic medium there would be created an osmotic gradient between body fluids and hindgut lumen resulting in body dehydration and swelling of the hindgut lumen. The opposite gradient would result if a hypotonic medium were drunk by the embryo. The question remains as to why this does not happen in the adult.

Summary

1. The osmoregulatory abilities of adult and embryonic *Cymatogaster aggregata* were tested by determining serum freezing points and weight changes after exposure to dilutions of sea water ranging between 15 and 100 per cent.

2. Adults were able to regulate in external media ranging between 20 and 100 per cent sea water. The greatest deviations of weight and serum freezing point from the normal occur after one to two days in the experimental media. Regulation in all media is complete at or before nine days of exposure.

3. The ability of embryos to regulate is proportional to their stage of development. The youngest animals tested regulate only in media with values between 25 and 36 per cent sea water.

4. Similar though less extensive experiments were performed with adult *Amphistichus argenteus* and *Micrometrus minimus*. The osmoregulatory ability of these species is identical to that of adult *C. aggregata*.

5. Preliminary experiments using Cl^{36} as a tracer indicate that the branchial salt secretory mechanism present in the adults is absent or operating less efficiently in the embryos of *C. aggregata*.

6. Observations on the volume of the gut in different external media indicate that the embryos drink the medium, thus creating an osmotic gradient between the body fluids and the lumen of the gut. The result is dehydration or "edema," depending upon the tonicity of the external medium.

LITERATURE CITED

Eigenmann, Carl H., 1890. The development of *Micrometrus aggregatus*, one of the viviparous serf perches. *Amer. Natur.*, **23**: 923–927.

Eigenmann, Carl H., 1894. *Cymatogaster aggregatus* (Gibbons): A contribution to the ontogeny of viviparous fishes. *Bull. U. S. Fish Comm.*, **12**: 401–478.

Gross, Warren J., 1954. Osmotic responses in the sipunculid worm *Dendrostomum zostericolum*. *J. Exp. Biol.*, **31**: 402.

Hubbs, Carl L., 1917. The breeding habits of the viviparous perch, *Cymatogaster*. *Copeia*, **47**: 72–74.

Smith, H. W., 1930. Absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.*, **93**: 480–505.

Tarp, Fred H., 1952. A revision of the family Embiotocidae (surf perches). Fish Bull. No. 88, California Div. Fish and Game, 99 p.

White, Philip R., 1954. The Cultivation of Plant and Animal Cells. The Ronald Press Co., New York, 239 p.

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